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Identification of novel glycosylation patterns in cancer and their role in cancer progression and metastasis

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Abstract

Aberrant glycosylation patterns are responsible for a significant increase in tumour malignancy. It is increasingly evident that such patterns play an important role in cancer progression, namely in invasion and metastasis, which is related to poor prognosis in cancer.

The expression of sialofucosylated glycans, such as sialyl-Lewis x and sialyl-Lewis a ($sLe^{x/a}$), by circulation tumour cells, seems to be essential and able to promote extravasation and tissues invasion, a crucial step in metastasis.

In this thesis, we aimed to identify the expression of aberrant glycosylation patterns, namely the $sLe^{x/a}$ antigens, in colon adenocarcinoma and TNBC tissues, using the immunohistochemical method, and then, using cell lines as experimental model, we have attempted to study the role of these patterns, through the inhibition of fucosylation, in cancer progression and metastasis.

Our results show that inhibition of fucosylation, using 2-fluorofucose inhibitor, is responsible for decreasing the expression of sialofucosylated ligands, such as the minimal determinant for E-selectin binding, the $sLe^{x/a}$, which is expressed in tumour tissues and decorate functionally important glycoproteins, being the Mucin 1 a candidate to carry this glycosylation pattern. Immunofluorescence assays show that this decrease seems to interfere with the cytoskeleton components, namely the cytokeratins, whose expression is increased. In addition, inhibition of fucosylation was also responsible for decreasing the expression of fibronectin, an important component of the extracellular matrix with functions in fundamental processes in cancer, such as the epithelial-mesenchymal transition. These phenotypic alterations can decrease the proliferative capacity and tumour cells migration, as well as, interfere with signalling pathways by modelling the expression of growth factors involved in the cell cycle progression, tumour growth, angiogenesis and invasion, as well as proinflammatory cytokines, including IL-1β and IL-6, whose expression was assessed by RT-qPCR.

Our results also suggest that the inhibition of fucosylation, besides reducing the expression of aberrant glycosylation patterns, can induce a set of morphological and phenotypic alterations that may lead to the restoring of a less aggressive phenotype considering invasion and cancer metastasis.

These features may be explored in the future to the study and development of new therapies that target the initial stages of metastasis, like the epithelial-mesenchymal transition, and fucosylation.

Keywords:

Fucosylation | Cancer | $sLe^{x/a}$ | EMT | Metastasis

Resumo

A Glicosilação consiste na adição enzimática que glicanos a estruturas não glicídicas, como as proteínas ou os lípidos, onde estão envolvidas enzimas como as glicosiltransferases e as glicosidases. É uma das principais modificações pós-traducionais nos eucariotas, ocorre ao longo da via Reticulo Endoplasmático-*cis/trans* Golgi e consiste num processo altamente regulado de onde resulta uma extensa diversidade de glicoconjugados. A esta grande diversidade está subjacente uma grande variedade de glicanos que resulta dos diferentes arranjos de açúcares e das diferentes ligações que estabelecem entre si para formar estruturas ramificadas e mais complexas. Existem vários tipos de glicosilação, no entanto, nas proteínas, a N e a O-glicosilação são os mais comuns. Estes dois processos diferem essencialmente no local de ligação à proteína, isto é, no aminoácido ao qual irá ficar ancorado o glicano, e no processamento das estruturas ramificadas.

Cada célula ou tecido, bem como diferentes estágios do desenvolvimento celular, têm uma identidade própria no que se refere aos glicanos que são produzidos. Por sua vez, os glicanos desempenham diversas funções nos mais diversos processos biológicos. Por exemplo, existem glicoproteínas, ou seja, proteínas às quais estão ligados glicanos, com funções fundamentais ao nível do reconhecimento celular, da adesão, da sinalização e até mesmo do próprio *folding*. Uma proteína pode transportar mais do que um padrão de glicosilação.

Tendo em conta a enorme importância dos glicanos nas funções biológicas, é natural que alterações nos processos de glicosilação sejam responsáveis por comprometer o correto funcionamento dessas mesmas funções ao nível celular, o que muitas das vezes resulta, por exemplo, em defeitos ao nível da adesão celular, com comprometimento da homeostase e do funcionamento dos tecidos. Assim sendo, a expressão de padrões de glicosilação aberrantes está associada ao cancro e é responsável por um aumento da sua malignidade, o que se traduz num rápido avanço na progressão tumoral e na formação de metástases, a principal causa de morte em cancro. Desta forma, defeitos nos padrões de glicosilação estão relacionados com um mau prognóstico em diversos tipos de cancro, entre eles, o cancro de colon, mama e pulmão.

Um dos padrões de glicosilação fortemente associado ao aumento da malignidade tumoral e da metástase é o sialil-Lewis x/a, na maioria das vezes ancorado a glicoproteínas que também desempenham um papel fundamental na progressão tumoral. Por exemplo, a Mucina 1, cuja expressão aumentada e a sua glicosilação alterada, têm sido reportadas em vários tipos de cancro, em especial, no cancro de colon e no cancro da mama. O sialil-Lewis x/a é ainda fundamental no reconhecimento da E-seletina, uma glicoproteína transmembranar muito glicosilada com funções de adesão, cuja expressão é fortemente influenciada pelos indicadores de inflamação, como por exemplo citocinas pró-inflamatórias, entre as quais a IL-1β. A grande maioria dos ligandos de E-seletina expressam este glicano sialofucosilado que, ao reconhecer a E-seletina expressa nas células endoteliais, diminuem o rolamento das células tumorais, aumentam a sua adesão dando-se início à invasão dos tecidos por essas células. A fucose é fundamental neste processo de reconhecimento, bem como os aminoácidos adjacentes e a presença de cálcio. Neste processo estão também envolvidos outros fatores, como por exemplo, os fatores angiogénicos que induzem a proliferação das células endoteliais e o aumento da permeabilidade endotelial, facilitando a entrada, nos tecidos, das células tumorais em circulação. Também na transição epitélio-mesenquima estes padrões de glicosilação parecem desempenhar um papel importante. Este processo consiste num programa celular e molecular em que as células epiteliais vão progressivamente diminuindo a expressão dos seus marcadores epiteliais, como as citoqueratinas e as E-caderinas, e aumentando a expressão dos seus marcadores mesenquimas, nomeadamente, a fibronectina e a vimentina. Assim sendo, as células vão progressivamente perdendo a adesão, descolam-se das massas tumorais primárias e entram em circulação. Por conseguinte, a expressão aumentada de ligandos de E-seletina, bem como de sialil-Lewis x/a, está relacionada com o aumento da progressão tumoral, nomeadamente, da invasão e da metástase.

O estudo destes padrões de glicosilação aberrante é fundamental uma vez que estão associados a uma maior malignidade, a um mau prognóstico e, por conseguinte, a uma menor sobrevivência em cancro.

Nesta tese, temos como principais objetivos avaliar a expressão de padrões de glicosilação aberrante em tecidos de cancro, identificando novos biomarcadores como potenciais alvos terapêuticos, nomeadamente candidatos a proteínas *scaffold* que transportem esses padrões de glicosilação, e ainda, avaliar esses padrões em modelos experimentais, designadamente em linhas celulares, de forma a compreender a função desses glicanos, bem como da fucosilação, na progressão tumoral e na metástase.

Identificámos estes padrões de glicosilação expressos em tecidos de cancro de colon e de cancro de mama, e através dos ensaios nas linhas celulares, mostrámos que a inibição da fucosilação, utilizando o inibidor 2-fluorofucose, é responsável por diminuir a expressão dos mRNAs das fucosiltransferases (FUT II, FUT IV, FUT V, FUT X e FUT XI) envolvidas na síntese dos antigénios de Lewis, bem como dos próprios ligandos sialofucosilados, como o sialil-Lewis x/a, que revestem glicoproteínas importantes, do ponto de vista funcional, entre as quais a Mucina 1, que também mostrámos estar expressa em tecidos tumorais e com padrões de glicosilação aberrante associados. Mais ainda, esta diminuição parece interferir com os componentes estruturais do citoesqueleto, nomeadamente, as citoqueratinas, que fazem parte dos filamentos intermédios, e cuja expressão, não só é aumentada, como o padrão de expressão é diferente em diferentes linhas celulares de cancro, sugerindo que a fucosilação afeta de forma diferente, diferentes citoqueratinas, confirmando que estas proteínas são uteis como biomarcadores que permitem caracterizar células e estágios tumorais. Relativamente à Mucina 1, verificámos que a inibição da fucosilação aumenta a expressão da sua forma sub-glicosilada que por sua vez fica retida no citosol, confirmando o papel da fucosilação no transporte para a membrana e reforçando uma possível função da Mucina 1 ao nível das vias de sinalização intracelular.

Além disso, a inibição da fucosilação foi também responsável por diminuir a expressão de fibronectina, um importante componente da matriz extracelular, com funções em processos fundamentais em cancro como a transição epitélio-mesenquima. A fibronectina é um marcador mesenquimal cuja expressão fica diminuída, enquanto que as citoqueratinas, um marcador epitelial, aumentam a sua expressão em resposta à inibição da fucosilação. Desta forma, a inibição da fucosilação parece ser capaz de retardar a entrada das células tumorais no programa de transição epitélio-mesenquima.

Estas alterações fenotípicas são capazes de diminuir a capacidade proliferativa e a migração das células tumorais, bem como atuar ao nível das vias de sinalização através da modelação, por um lado, da expressão de fatores de crescimento envolvidos na progressão do ciclo celular, no crescimento tumoral, na angiogénese e na invasão, como os fatores TGF-β (*Transforming Growth Factor β*), FGF2 (*Fibroblast Growth Factor 2*) e VEGF-A (*Vascular Endothelial Grwoth Factor A*), e por outro, na expressão de citocinas pró-inflamatórias, entre as quais a IL-1β e a IL-6. Ou seja, o contexto ao nível do microambiente tumoral é alterado, por consequência do tratamento com o inibidor de fucosilação, o que tem implicações em duas das principais características das células tumorais, a proliferação e a migração, e por conseguinte, na progressão tumoral e na metástase.

No conjunto, os nossos resultados, além de confirmarem a importância dos ligandos de E-seletina ao longo do desenvolvimento tumoral, sugerem que a inibição da fucosilação, além de diminuir a expressão de padrões de glicosilação aberrantes, neste caso o sialil-Lewis x/a, é capaz de induzir um conjunto de alterações morfológicas e fenotípicas, nomeadamente ao nível das citoqueratinas, da fibronectina e da Mucina1, que poderão conduzir ao restauro de um fenótipo menos agressivo no que diz respeito ao desenvolvimento tumoral, nomeadamente a invasão e a metastização tumorais, ou seja, é capaz de retardar a progressão tumoral.

Uma melhor compreensão destas características e propriedades poderão vir a ser exploradas no estudo e no desenvolvimento de novas terapias que tenham como alvo terapêutico as fazes iniciais da metastização, como a transição epitélio-mesenquima, e a fucosilação.

Palavras Chave:

Fucosilação | Cancro | $sLe^{x/a}$ | EMT | Metástase

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List of abbreviations

2-FF – 2-Fluorofucose

APC - Allophycocyanin

Asn – Asparagine

BSA – Bovine Serum Albumin

Ca^{2+} - Calcium ion

cDNA – complementary Deoxyribonucleic Acid

CFSE - Carboxyfluorescein Succinimidyl Ester

CK – Cytokeratin (s)

CR – Consensus Repeats

CRC – Colorectal cancer

C_t – Threshold cycle

DAB - 3,3'-Diaminobenzidine

DAPI - 4',6-diamidino-2-phenylindole

DMEM - Dulbecco's Modified Eagle Media

DMSO - Dimethyl sulfoxide

dNTP (s) – Deoxynucleotide (s)

ECM – Extracellular Matrix

EDTA - Ethylenediaminetetraacetic acid

EGF – Epidermal Growth Factor

EGFR - Epidermal Growth Factor Receptor

EMT – Epithelial-Mesenchymal Transition

ER – Endoplasmic Reticulum

E-SL – E-selectin Ligands

FBS – Fetal Bovine Serum

FITC - Fluorescein

Fuc - Fucose

FUT (s) – Fucosyltransferase (s)

Gal – Galactose

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

Glc - Glucose

GlcNAc – N-Acetylglucosamine

GT (s) – Glycosyltransferase (s)

h – hour

HRP – Horseradish peroxidase

IDC – Invasive Ductal Carcinoma

IF – Immunofluorescence

Ig (s) – Immunoglobulin (s)

IHC - Immunohistochemistry

IL- Interleukin

kDa - kilodalton

$Le^{a/b/x/y}$ – Lewis a/b/x/y antigen

mAb (s) – Monoclonal antibody (ies)

Man – Mannose

MET - Mesenchymal-Epithelial Transition

MUC – Mucin

mRNA – Messenger RNA

n.d. – Not detected

Neu5Ac – N-acetylneuraminic acid

N-GalNAc – N-Acetylgalactosamine

NT – Not treated

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

ppGalNAcT – Acetyl-galactosaminyltransferase

PVDF - Polyvinylidene fluoride

qPCR – quantitative Polymerase Chain Reaction

RNA – Ribonucleic Acid

RT – Room temperature / Reverse Transcriptase

SDS-PAGE – Sodium dodecyl sulfate – polyacrylamide gel electrophoresis

Ser- Serine

$sLe^{x/a}$ – sialyl-Lewis x/a

ST (s) – Sialyl1transferase (s)

ST3GAL (s) – β-galactoside α2,3 sialyltransferase (s)

STn – Sialyl-Tn

TACA – Tumour-associated Carbohydrate Antigens

TBST – Tris-buffered Saline Solution-Tween

TGF-β - Transforming Growth Factor – β

Thr – Threonine

TNBC – Triple Negative Breast Cancer

TNF-α - Tumour Necrosis Factor α

VNTR - Variable Number of Tandem Repeats

WB – Western blot

WHO – World Health Organization

1. Introduction

1.1. Glycosylation

Carbohydrates are one of the organic macromolecules that compose living systems[1]. The surface of eukaryotic cells is decorated externally by a dense matrix of glycosidic structures – the glycocalyx, with important biological functions[2]. Generally, we use the word glycan to describe free or covalently bound saccharide residues which form the glycoconjugates[3]. The great glycan's structural diversity results from the different types of sugars that compose them, and the different types of bonds established between them[4]. Each type of cell and tissue has its own identity with regard to glycans which are expressed, in addition, certain patterns of glycosylation are characteristic of certain stages of development and cell or tissue differentiation[5]. We call Glycome to the set of all glycans that can be synthesized by an organism[6]. Glycans are involved in several cellular functions since structural stability to cell-cell and cell-matrix interactions, as well as cell signalling and even in maintenance of protein folding[7,8,9,10].Glycosylation is not template driven, but may be considered as secondary gene products. In fact, its synthesis is dependent on the expression of genes that encode for certain enzymes and carriers[11].

Glycosylation is one of the main post-translational modifications in eukaryotes and consists in the enzymatic addition of glycans to non-glycosidic structures such as proteins and lipids[12,13]. The glycosyltransferases (GT) are key enzymes in glycans' biosynthesis, they act sequentially and are specific to the sugar donor, but can recognize different acceptors, they are classified depending on the sugar they transfer[14]. Glycosidases are also important in the glycosylation process, these enzymes are involved in the removal of sugars during the processing of glycans[15]. Glycosylation occurs essentially in the Endoplasmic Reticulum (ER) and in the Golgi Apparatus, however, important modifications also occur in the cytoplasm, the cell membrane and in the extracellular matrix (ECM)[16]. Most of the GTs and the glycosidases are associated to the ER – *cis/trans* Golgi network[17], and their expression levels, location and specificity are factors that determine glycosylation patterns[18].

1.1.1. N-glycosylation and O-glycosylation

Proteins containing one or more covalently linked glycans to their polypeptide chains are designated as glycoproteins[13]. There are two fundamental types of protein glycosylation[12]: N-glycosylation, where are produced glycans linked to the amide group of the Asparagine residue (Asn) in the lateral chains of the proteins, and O-glycosylation where glycans are bound to the hydroxyl group of Threonine (Thr) or Serine (Ser) residues[19,20]. Generally, and unlike the O-glycans, the N-glycans are larger and processed structures[21] (Figure 1.1). Different types of glycosylation can be present in the same glycoprotein[22].

Focusing on N-glycosylation, about 90% of eukaryotic glycoproteins are N-glycosylated[23]. The biosynthesis of N-glycans starts at the ER with the formation of an oligosaccharide precursor bounded to the dolichol phosphate, a lipid transporter initially on the cytosolic face of the reticular membrane, where, before turning to the reticular lumen, N-Acetylglucosamine (GlcNAc) and Mannose (Man) residues are added. Once on the luminal side, Glucose (Glc) is added, and the precursor is transferred in block, by the action of oligosaccharyltransferases, to a nascent protein, forming an N-glycosidic bond between a GlcNAc and an Asn residue that integrates a consensus sequence Asn-X-Ser/Thr (where X can be any amino acid except Proline)[24]. In the next step, the N-linked glycan is processed by the action of glycosidases and GTs to form branched and terminal structures. First, some residues are removed –

trimming, and then others are added – extension. This step takes place initially in the ER and later in the Golgi[25,26].

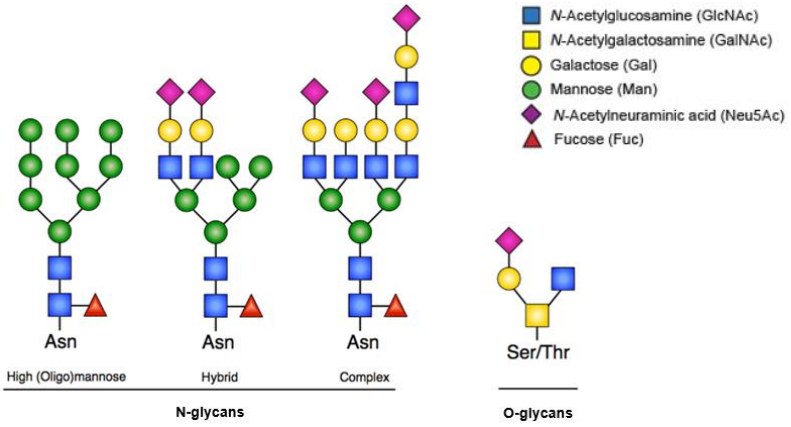

Figure 1.1 – N-glycan and O-glycan structures. N-linked glycosylation occurs through consensus Asn residues whereas O-linked glycosylation occurs through Thr or Ser amino acids. Three different N-glycans share a common *core* structure. Adapted from Lyons, Milner and Rosenzweig (2015)[27].

In O-glycosylation, the biosynthesis of O-glycans occur in Golgi by the sequential action of different GTs, whose arrangement influences the pattern of the O-glycan produced[28]. The first step consists in the transfer and attachment of N-Acetylgalactosamine (N-GalNAc) to the hydroxyl group of the amino acid Thr or Ser in the protein by the action of Acetyl-galactosaminyltransferase (ppGalNAcT) forming the simplest structure, the Tn antigen, from which the chains are extended by the action of GTs and forming more complex structures[29]. During chains extension, even structures common to N-glycosylation can be formed, for example the sLe$^{x/a}$ [30], this epitope will be better characterized in section 1.3.1.

In cancer, the sialylation is increased[31] and the Tn antigen can be modified, forming the Sialyl-Tn (STn) antigen[32]. Short O-glycan structures are common features in cancer[33].

1.2. Cancer

According to the World Health Organization (WHO), cancer is one of the leading causes of death in the world, having been responsible for 8.8 million deaths in 2015 and 9.6 million people worldwide are estimated to die from cancer in 2018[34]. In Portugal, the incidence of cancer has increased about 3% per year and was responsible for the deaths of about 27000 patients in 2015[35]. It is estimated that in 2030 Portugal may reach 60000 new cases of cancer. Among the most lethal cancers are lung, colorectal, stomach, prostate and breast cancer[36]. Currently, between 30-50% of cancers can be prevented avoiding risk factors like tobacco, alcohol, diet and sedentary lifestyle and implementing strategies for prevention and early diagnosis[34].

Cancer cells break the rules by which multicellular organisms are built and maintained[37]. The gradual process of transformation of a normal cell into a cancer cell is called cancerigenesis.

Cancer begins with genetic mutations and epigenetic changes as a result of cell exposure to chemical, physical and biological carcinogenic agents[38]. These mutations can lead to activation of oncogenes and inactivation of tumour suppressor genes, cell proliferation and survival are altered, resulting in an uncontrolled division and reduction or inhibition of cell death. Cancer cells become immortal giving

rise to an accelerated increase of the cell population that continues to accumulate mutations[39]. Local tissues invasion follows as a result of changes in cell adhesion of primary tumour cells and then the spread of those cells to other sites through the circulatory and lymphatic system. Cancer cells are able to survive in circulation and colonize new tissues establishing new cellular interactions and forming metastasis that can become highly invasive. Metastasis consists in the formation of the tumour at new sites as a result of the migration of cancer cells from an initial focus[40]. Metastasis is the leading cause of death in cancer[41].

In 2010, the so-called Hallmarks of cancer, initially proposed by Hanahan and Weinberg in 2000, were reviewed. These consist of a set of acquired features that allow to characterize the cancer cells during tumour development. It was established as Hallmarks of cancer: sustained proliferative signalling, evade growth factors, resistance to cell death, unlimited replication capacity, reprogrammation of energy metabolism, angiogenesis induction, evading the immune response and tissues invasion and metastasis. Facilitating the establishment of all these features are the genomic instability and the inflammation as well as the tumour microenvironment that has given a new dimension and complexity to the cancerigenesis process[42].

1.3. Glycosylation and cancer

Aberrant cancer associated glycosylation patterns were first described more than 45 years ago[43]. In fact, during the tumour progression there are significant alterations in glycosylation patterns that are responsible for a significant increase in tumour malignancy[44]. It is clear that they play an important role in cancer progression, particularly in signalling pathways and metastasis[45,46,47]. Certain changes in glycosylation patterns are strongly related to prognosis in cancer.

There are several mechanisms that can lead to an aberrant glycosylation pattern also called tumour-associated carbohydrate antigens (TACA)[48]. These are characterized by the presence of incomplete or truncated glycans, the increase or loss of glycans expression or even the appearance of new structures. To these patterns are also associated changes in the levels of GTs expression and activity as well as changes in sugar transport molecules and consequent accumulation of precursors[4549].

Among the TACA we can distinguish the Lewis antigens, in particular sLe^x and sLe^a, which can be present in N- and O-glycans and the Thomsen-Freidenreich antigens, which are truncated O-glycans and include the sialylated form of the Tn antigen, the STn[48].

Considering the significant importance of glycosylation, alterations in their expression patterns compromise certain cellular functions, namely cell adhesion and cellular interaction with consequences in invasion and cancer metastasis[44,47]

1.3.1. Lewis antigens - Sialyl-Lewis A and Sialyl-Lewis X

Lewis antigens are terminal epitopes, normally on cell surface glycoproteins, which constitute a group of loading a α 1,3/4 fucose residue bound to GlcNAc. They are present in both N-glycans and O-glycans and are synthesized by N-acetylglucosaminyltransferases, galactosyltransferases, sialyltransferases (STs) and fucosyltransferases (FUTs)[50,51].

Structurally, they are tetrasaccharides with a Gal-β(1,3/4)-GlcNAc-R backbone. In type I chains (Le^a, sLe^a and Le^b) the bound is β1,3 while in type II chains (Le^x, sLe^x and Le^y) is β1,4. Both types contain a fucose (Fuc) residue bound to GlcNAc, transferred by FUTs. FUTs can be grouped into three subgroups, α1,2 FUTs, α1,3/4 FUTs and α1,6 FUTs. α1,3/4 FUTs are especially involved in the last stages of Lewis antigens synthesis[43,52]. In fact, in case of Lewis antigens, Fuc is α1,4 bound in type I and α1,3 in type II chains. In type I chain, the addition of a Fuc is catalysed by FUT III while in type II chain the FUTs III, IV, V, VI, VII and IX are involved. Finally, terminal galactose (Gal) can be sialylated or fucosylated. In the case of sialylation, which consists in the addition of a sialic acid residue (the N-acetylneuraminic acid (Neu5Ac) is the most common in humans), are involved STs, specially the β-galactoside α2,3 sialyltransferases (ST3GAL), with sLe^x and sLe^a formation. If galactose is fucosylated we have the synthesis of Le^b and Le^y by FUT I and FUT II (Figure 1.2)[50,53].

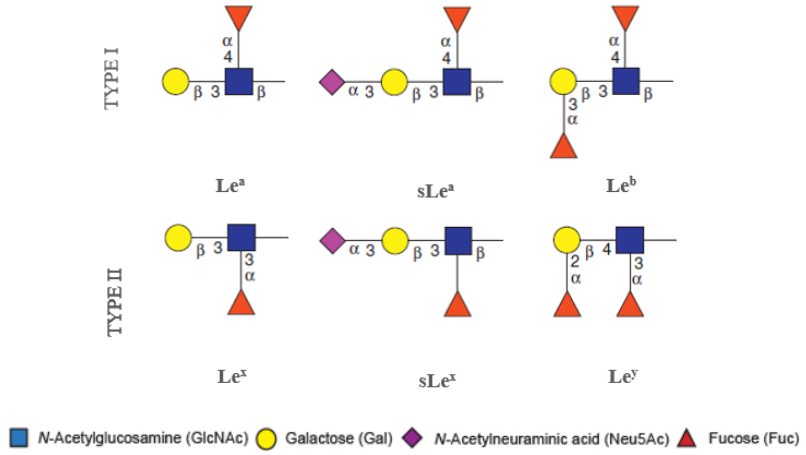

Figure 1.2 – Lewis antigens. They are a group of glycans that carry a Fuc linked to GlcNAc residue. In sialylated forms a sialic acid residue are added to terminal Gal residue. Adapted from Juge (2012)[54].

Increased expression of $sLe^{x/a}$ has been reported in certain types of cancer, such as colon and breast cancers, and this overexpression is associated with poor prognosis[50,55,56,57]. Sialylation and fucosylation have also been implicated in malignant transformation, especially the changes in the levels of STs and FUTs expression, which has been reported in some types of cancer[58,59,60].

1.3.2. Aberrant glycosylation and Metastasis

The extravasation of cancer cells to new tissues and distant organs is a fundamental step in tumour invasion and metastasis[40]. These cells use a process similar to the migration and extravasation of leukocytes, to invade and colonize new sites[61].

Circulating cancer cells need to slow down and create new interactions with endothelial cells in the tissues that they will colonize. Selectins and its ligands, as well as their glycosylation patterns, play an important role in the establishment of these interactions[62,63].

1.3.2.1. sLe$^{x/a}$ as E-selectin ligands

The selectins are glycoproteins present on the cell surface with adhesion functions. They are a family of three calcium-dependent lectins (Type C) which, in addition to transmembrane domain and a short C-terminal cytoplasmic tail, have an N-teminal lectin domain, an Epidermal Growth Factor (EGF)-like domain and a variable number of Consensus Repeats (CR)[64]. This structure is highly conserved in mammals[65]. Depending on where they are expressed, the selectin is called E-selectin (endothelium), L-selectin (lymphocyte) or P-selectin (platelets)[64].

E-selectin (CD62e, ELAM-1, LCAM-2), expressed in the activated endothelium, are highly glycosylated transmembrane glycoproteins whose interactions with their sialofucosylated ligands, proteins or lipids, results in decreased rolling and increased cell adhesion, with the establishment of new cell-cell interactions, where Integrins are also involved[66,67]. The expression of E-selectin, also a marker of inflammation, is induced by proinflammatory cytokines such as Interleukin-1β (IL-1β) and Tumour Necrosis Factor α (TNF-α)[68,69]. Cancer cells are able to secrete proinflammatory cytokines, generating inflammation sites[42] and inducing the expression of E-selectin in endothelia, which in turn recognize their ligands expressed on cancer cells surface. So, cancer cells are recruited and the transendothelial migration and adhesion cascade begins[67].

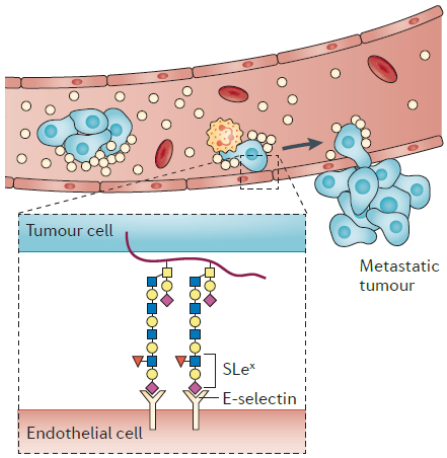

Figure 1.3 – sLe$^{x/a}$ in cancer progression and metastasis. sLe$^{x/a}$ expressed in tumour cells plays a role in extravasation and tissue invasion by recognizing receptors, namely E-selectin, expressed in endothelial cells. Adapted from Pinho and Reis (2015)[44].

E-selectin ligands (E-SL) are generally transmembrane glycoproteins with sialylated and fucosylated structures[64]. sLe^x and sLe^a are two recognizing E-selectin motifs and are part of the proteins and lipids scaffolds able of binding to E-selectin. Fuc, which interacts with calcium ion (Ca^{2+}) and some amino acids near the E-selectin lectin domain, is an essential component for recognition and binding to E-selectin[65,70] (Figure 1.3).

Thus, increased expression of sLe$^{x/a}$, as well as E-SL, in tumour cells is strongly related to the metastatic potential and tumour growth and invasion, being these indicators of malignancy and poor prognosis[71,72]. In addition, the increase of E-selectin expression by endothelial cells near metastasis sites also facilitates the extravasation of these cancer cells and tissues colonization[64,66].

1.3.2.2. Glycoproteins as E-selectin ligands in Cancer

For E-selectin recognition, besides the glycans structure, also the scaffold protein decorated with this glycan plays an important role[73]. In fact, the role of $sLe^{x/a}$-decorated glycoproteins has been reported in cancer[65,70].

Mucins are a group (MUC1-9) of large glycoconjugates that correspond to strongly O-glycosylated and moderately N-glycosylated proteins that can be soluble, secreted or transmembrane[74]. When present in the membranes, they fill the apical surfaces of epithelial cells, especially in the respiratory and gastrointestinal tract, where their distribution follows a specific pattern that characterize these tissues. They protect the organs from infections[75]. Mucins can exhibit aberrant glycosylation[76] patterns and play an important role in cancerigenesis[77].

Mucin 1 (MUC1) is an important cancer associated antigen that is overexpressed in some types of adenocarcinoma, like colon and breast cancer[76] and is also implicated in binding to E-selectin[78]. O-glycosylation of MUC1 is well characterized in several cell types and tissues unlike N-glycosilation that occurs only in five sites, where just one is extracellular, and which seems to be important for their folding and transport[79]. In tumour context, the expression of an altered MUC1 interferes, not only with its biochemical functionality, but also with its distribution[76]. In cancer cells, MUC1 also participates in the signal cascades regulating the expression of target genes. For example, MUC1 interacts with β-catenin through phosphorylation of its cytoplasmic tail. Overexpression of MUC1 also attenuates apoptosis through Akt (Protein kinase B) or p53 pathways[80].

MUC1 also appears to be implicated in the triggering of the epithelial-mesenchymal transition (EMT) as a result of biochemical changes that lead to loss of cell polarity that increases tumour invasiveness and metastatic potential[76,80,81].

1.4. Epithelial-Mesenchymal Transition (EMT), Invasion and Metastasis

The EMT is a molecular and cellular developmental regulatory program in which cells that are initially part of tissue layers acquire the ability to move[82]. Cancer cells are able to use part of this transition mechanism for epithelial cells of primary tumours undergo morphological and phenotypic changes, such as loss of adhesion, and acquire a migratory capacity that allows them to invade distant tissues and organs, forming metastasis[83]. Thus, cells submitted to EMT appear to be intended to migrate[42].

This transition mechanism, induced by growth factors like Transforming Growth Factor – β (TGF-β), involves changes in the expression of certain genes, namely the activation of genes encoding for mesenchymal markers, such as Vimentin and Fibronectin, and downregulation of epithelial markers like Cytokeratins (CK), an important component of intermediate filaments, E-cadherins and MUC1[82,84].

These alterations lead cytoskeleton reorganization and consequent changes in cell polarity. In addition, there are loss of epithelial adhesion and synthesis of components that modify the composition of ECM, affecting tissue structure and cellular communication. Thus, EMT is associated with increased metastatic potential[85].

Aberrant glycosylation is also involved in EMT since the expression of certain patterns influences cell adhesion, i.e., they directly influence cellular communication mechanisms, namely in the signal transduction and in the activation of cellular receptors[86]. Moreover, there are studies showing that

fucosyltransferases, namely FUT IV, activate signalling systems that induce the transcription of mesenchymal markers related genes[87]. Also alterations in the regulation of sialylation were reported during EMT, which showed to influence the TGF-β sialylation state[88].

Fibronectin is a cell-matrix adhesion glycoprotein that bind to the Integrins present on the cell surface, performing an evident structural function[89]. It is expressed in several types of cancer and seems to be involved in several stages of tumour development[90]. In fact, increased expression of fibronectin in cancer has been related to the promotion of tumour growth[91], migration[92] and invasion[93]. Moreover, being an adhesion protein, influences several signalling events that lead to an increased metastatic potential[93,94].

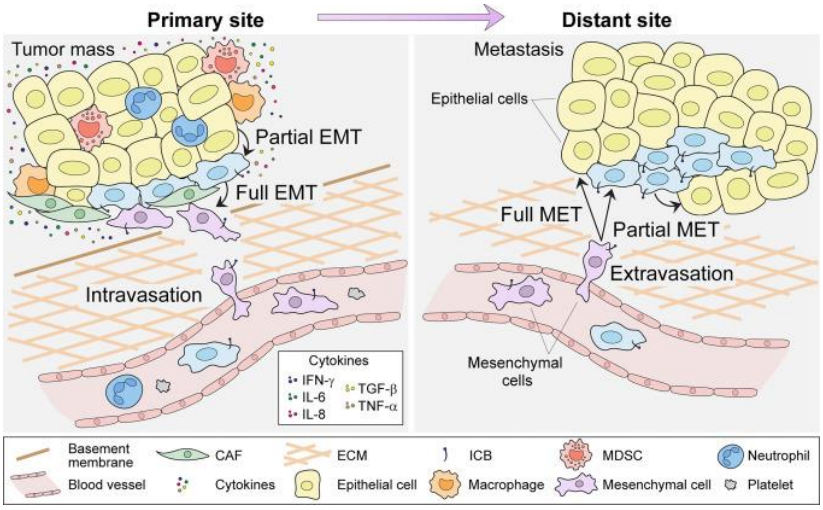

Figure 1.4 – Epithelial-Mesenchymal Transition network. Primary tumour cells start to increase the expression of mesenchymal markers due to the presence of proinflammatory cytokines in the tumoral microenvironment where CAF, MDSC, Macrophages and infiltrated Neutrophils are also present. After undergoing in EMT stage, cancer cells can degrade ECM, go into bloodstream and migrate do distant sites, where, through E-selectin interactions, they colonize new tissues, start do increase the expression of epithelial markers and form new tumour masses, the metastasis. Taken from Sistigo and Nisticò (2017)[95]. CAF – Cancer-associated fibroblast; MDSC – Myeloid-derived suppressor cell; ICB – Immune checkpoint blocker; MET – Mesenchymal-epithelial transition;

1.5. Introduction to the aims of this thesis

Aberrant glycosylation patterns are responsible for a significant increase in tumour malignancy. Therefore, in this thesis, we hypothesized that increased expression of $sLe^{x/a}$ in cancer tissues induces more aggressive features in cancer. So, we have as main objectives to evaluate the expression of aberrant glycosylation patterns in cancer, identifying novel biomarkers as potential therapeutic targets, namely scaffold protein candidates, and evaluate these patterns in experimental models seeking to understand the role of these glycans in cancer progression and metastasis.

At first, we started by evaluating the expression of $sLe^{x/a}$ and certain biomarkers in colon adenocarcinoma and TNBC tissues by Immunohistochemistry (IHC). After this characterization, we went to evaluate the expression of these glycans in cell lines and study their role in cancer progression and metastasis, by inhibiting fucosylation and assessing its effects on gene expression and fundamental processes in cancer, such as cell proliferation and migration, using Immunofluorescence (IF), flow cytometry and Western blot (WB) assays. Finally, we evaluated the role of fucosylation in EMT.

2. Materials and Methods

2.1. Histological analysis

Formalin fixed paraffin-embedded colon and breast cancer tissues were sectioned and placed onto slides (Thermo Scientific) using standard paraffin microtomy. The Novolink™ Polymer Detection System from Leica BIOSYSTEMS was used for all procedures. All the steps of this protocols took place at room temperature (RT).

Slides were deparaffinized in xylene, washed in graded ethanol and rehydrated in de-ionized water. For antigen retrieval, slides were incubated in a microwave for 30 minutes using a citrate buffer pH 6 and thereafter washed with tap water. Endogenous peroxidase activity and proteins were blocked by incubating slides with Peroxidase block and Protein block solutions, respectively, for 5 minutes and then rinsed three times with Tris-Buffered Saline solution with 0.1% Tween 20 (TBST) (Appendix 1).

The slides were incubated with HECA-452 mAb (1:50) (BioLegend®), anti-CD15s mAb (1:50) (BD Biosciences), anti-CA19.9 mAb (1:50) (Abcam), anti-CK – clone AE1/AE3 (1:100) (Dako), anti-MUC1 – clone VU4H5 (1:100) (Santa Cruz), anti-MUC1 – clone 5E3 (1:100) (homemade) or anti-Ki67 (1:20) (BioLegend®) diluted in Diamond Antibody Diluent (Cell Marque) for 45 minutes, followed by incubation with Post primary and Novolink™ Polymer solutions for 30 minutes. Slides were rinsed three times with TBST between each incubation. For E-selectin staining with E-selectin-human Ig Fc chimera (E-Ig) (R&D Systems) an additional step was made. After incubation with E-Ig (1:300), slides were incubated with anti-CD62e mAb (1:250) (BD Biosciences) also diluted in Diamond Antibody Diluent for 30 minutes. In this specific case, all solutions in the staining process had 2 mM of Calcium chloride ($CaCl_2$). Chromogenic detection was performed by incubating slides with 3,3'-Diaminobenzidine (DAB) Chromigen and Novolink™ DAB Substrate Buffer (1:20) for 4 minutes. Positive staining produces a dark brown reaction product. Immediately after the colour development, slides were washed with tap water for 2 minutes. Nuclear contrast staining was performed with hematoxylin (Bio-Optica) for 1 minute. Slides were washed with tap water in order to blueing hematoxylin stain. After the immunohistochemical technique, the slides were dehydrated, treated with increasing concentrations of alcohol, cleared in xylene and mounted in Quick-D-M-Klinipath mounting medium.

Slides were visualised after digital scanning using NanoZoomer-SQ Digital Slide scanner (HAMAMATSU) and NDP.view2 Viewing software (HAMAMATSU). For evaluation, a semi-quantitative approach was established. Cell staining was quantitatively evaluated as 0 when negative, 1 if [1-25]%, 2 if]25-50]%; 3 if]50-75]% and 4 if]75-100]% cells were stained and staining intensity was evaluated as absent (0), weak (1), moderate (2) and strong (3). The score was obtained by multiplying each of the two quantitative values, the % of stained cells and the staining intensity. For Ki-67 analysis, a random area was selected, 100 cells were counted, and the percentage of stained cells calculated.

2.2. Cell culture

CF1T and HT29, LS174T and SW48 cell lines were used as *in vitro* models of breast cancer and colorectal adenocarcinoma, respectively. HT29 (ATCC®HTB-38™) cell line was derived from a 44-year-old Caucasian female colon adenocarcinoma. LS174T (ATCC®CL-188™) cell line was established from Dukes' type B adenocarcinoma of colon from a 58-year-old Caucasian female. SW48

(ATCC®CCL-231™) cell line was derived from Dukes' type C 82-year-old Caucasian female colon adenocarcinoma. Finally, CF1-T cell line was established from Invasive Ductal Breast Carcinoma (IDC) as described by Carrascal et.al[96].

The cell lines used were cultured in T25 culture flasks (SARSTEDT) at 37ºC with 5% Carbon dioxide (CO_2) and a humidified atmosphere. CF1T, HT29 and LS174T cells were cultured with Dulbecco's Modified Eagle Media (DMEM) (Gibco®, Life Technologies) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Gibco®, Life Technologies), 1% (v/v) L-glutamine (Gibco®, Life Technologies) and 1% (v/v) Penicillin/Streptomycin (Gibco®, Life Techonologies). SW48 cells were cultured with Liebovitz L-15 medium (Sigma-Aldrich) supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine and 1% (v/v) Penicillin/Streptomycin. The medium was changed every 3-4 days. For passages, at 80% of confluency, the culture was washed with 1X Phosphate Buffered Saline (PBS) (Appendix 1) and trypsinized for 5 minutes with 1X trypsin-Ethylenediaminetetraacetic acid (EDTA) (Gibco®, Life Technologies) for cell detachment, followed by a centrifugation step (Eppendorf) at 200*g* for 5 minutes and sub-cultured according to the desired dilution. All cell lines were stored at -80ºC resuspended in culture medium with 10% (v/v) Dimethyl sulfoxide (DMSO) (Sigma Aldrich).

To determine the cell number necessary for each assay a Neubauer counting chamber (Marienfeld) was used.

2.3. 2-Fluorofucose treatment

Cell lines was treated or not with 1 mM of 2-Fluorofucose (2-FF) inhibitor in supplemented culture medium. Cells must be exposed to the inhibitor for at least 5 days.

2.4. Flow cytometry

For each condition, $3x10^5$ cells were collected, washed with 500 µL of PBS (Appendix 1) and centrifuged at 200g for 5 minutes. Supernatant was discarded, and cells were washed again with 900 µL of PBS and centrifuged at 200g for 5 minutes. Pellets were resuspended in the desired volume of PBS to be divided as 100 µL in each condition. Expression analyses of $sLe^{x/a}$ was performed using HECA-452 mAb (0.5 µL) for 30 minutes at 4ºC followed by a washing step with 500 µL of PBS and centrifugation at 200g for 5 minutes. Supernatant was discarded, and the cells were incubated with anti-rat IgM-APC secondary antibody (0.5 µL) (BD Pharmingen) at RT for 15 minutes. Next, cells were washed with 500 µL of PBS and centrifuged at 200g for 5 minutes. The supernatant was discarded, and pellets resuspended in 1 mL of PBS. The same procedure was used for MUC1 expression analysis but using anti-MUC1 – clone VU4H5 mAb (1.5 µL) followed by anti-mouse Ig-FITC secondary antibody (1.5 µL) (Dako). For intracellular staining, to assess the expression of CK, we used the Fixation/Permeabilization Solution Kit (BD Bioscience), according to manufacturer's instructions, before staining cells with anti-CK – clone AE1/AE3 mAb (1.5 µL) for 30 minutes at 4ºC followed by incubation with anti-mouse Ig-FITC secondary antibody (1.5 µL) for 15 minutes at RT. The E-SL expression was analysed incubating cells, after washing, with E-Ig chimera (0.5 µg/µL) plus anti-human Ig-FITC antibody (1 µL) (Sigma-Aldrich) in PBS-Ca^{2+} or, as negative control, in PBS with 2mM EDTA for 1 hour at 4ºC. After that, cells were washed with 1 mL of corresponding PBS and centrifuged at 200g for 5 minutes. The supernatant was discarded, and cells resuspended in 1 mL of PBS-Ca^{2+} or PBS-EDTA. Control assays were performed incubating cell with only fluorescent-labelled secondary antibodies. Flow cytometry was performed in Attune® Acoustic Focusing Cytometer (Applied Biosystems), at least $1x10^4$ events were acquired and results were analysed using FlowJo v.10 software.

2.5. Immunofluorescence

Cells were cultured on round coverslips inside 24 well plates (Orange Scientific) for 24 hours. Then, cells were washed three times with PBS (Appendix 1) for 5 minutes and fixed and permeabilized with 200 μL of Fixation/Permeabilization solution, for 20 minutes at 4ºC. After three washes with PBS for 5 minutes, cells were blocked with 1% Bovine Serum Albumin (BSA) (Sigma-Aldrich) and washed three times again. Next, cells were subject to immunofluorescence staining with HECA-452 mAb (1:50), anti-MUC1 – clone VU4H5 mAb (1:200), anti-CK – clone AE1/AE3 mAb (1:200) or anti-Fibronectin mAb (1:200) during 30 minutes at RT followed by three washes with PBS. Cells were incubated with anti-rat IgM-FITC (1:50) (BD Pharmingen), anti-mouse IgG-FITC (1:100) (Dako) or anti-mouse Ig-FITC (1:100) secondary antibodies for 30 minutes at RT and washed three times with PBS. Negative controls were performed staining with only fluorescent-labelled secondary antibodies. All antibodies were diluted in 1X BD Perm/Wash buffer. Finally, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (1μg/mL) (ThermoFisher) for 10 minutes in the dark, washed, mounted with 20 μL of montage medium Mowiol+DABCO and analysed by fluorescence microscopy. Fluorescence intensities from five randomly selected microscopic fields of cells were quantitatively analysed with ImageJ software and using CTCF formula (2.1):

(2.1) CTCF = Integrated density – (Area of selected cell x Mean fluorescence of background readings)

2.6. SDS-PAGE and Western blot

Cells were lysed in IP Lysis/Wash buffer (Thermo Scientific) complemented with 1% Protein Inhibitors (Roche) and incubated for 20 minutes. Every 5 minutes, the suspension was vortexed for 1 minute. Supernatants were cleared by centrifuged at 10000g for 10 minutes and protein concentration was quantified using Pierce BCA Protein Assay Kit (Thermo Scientific) following manufacture's instructions. After that, samples were loaded in 4% stacking and 6% resolving gel (Appendix 1) for $sLe^{x/a}$ and MUC1 analysis, or 12% resolving gel (Appendix 1) for CK analysis and the electrophoresis was performed in Running buffer (Appendix 1) at 100 V. Sodium dodecyl sulfate – polyacrylamide gel electrophoreses (SDS-PAGE) resolved proteins were transfer into a polyvinylidene fluoride (PVDF) membrane at 400 mA for 1 hour at 4ºC and using Transfer buffer (Appendix 1). Then, membranes were blocked with 7,5% non-fat milk powder dissolved in TBST 0.1% (Appendix 1) for 1 hour at RT. Membranes were incubated with HECA-542 mAb (1:1500), anti-CK – clone AE1/AE3 (1:200) or anti-MUC1 – clone VU4H5 (1:200) diluted in TBST 0.1% overnight at 4ºC. After three washes of 10 minutes in TBST 0.1%, membranes were incubated with appropriated Horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in TBST 0.1% for 1 hour at RT. Membranes were washed again and Lumi-light Western Blotting Substrate (Roche) was used for detection. At the end, the revelation was performed exposing the membranes to Amersham Hyperfilm ECL (GE Healthcare Life Sciences) and using Corestream® Kodac® autoradiography GBX developer and Corestream® Kodac® autoradiography GBX fixer (sigma).

2.7. Gene expression analysis – RT-qPCR

First, total Ribonucleic acid (RNA) was extracted using GenElute™ Mammalian Total RNA Purification kit (Sigma-Aldrich), according to manufacturer's instructions. Optional on-column DNaseI digestion (Qiagen) was also performed. Next, 10 μL of purified RNA was reverse transcribed using

High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The conversion mix was prepared by adding, for each PCR tube (VWR), 2 µL of RT Buffer, 2 µL of RT Random Primers, 0.8 µL of deoxynucleotides (dNTPs), 0.25 µL of MultiScribe Reverse Transcriptase enzyme and 4.2 µL of RNase free water (NZYTech). Complementary Deoxyribonucleic acid (cDNA) conversion was performed in a thermocycler (Programmable Thermal Controller PTC-100™ – MJ Research) with the following program: Step I -10 minutes at 25ºC, Step II - 120 minutes at 37ºC and Step III - 5 seconds at 85ºC. Real-time PCR was performed starting with preparing the RT-PCR mix, adding for each RT-PCR tube (Simport), 2 µL of cDNA, 2 µL of diluted probe 1:4, 1 µL of RNase free water and 5 µL of the Master Mix Fast (Applied Biosystems). The probes used were FUT II, FUT IV, FUT V, FUT X, FUT XI, TGF-β, FGF2, VEGF-A, IL-1β and IL6 (Applied Biosystems). All experiments were performed in duplicates and two endogenous controls were used, β-actin and Glyceraldehyde 3-phosphate dehydrogenase (GADPH). Samples were run in RotorGene 600 (Corbett) with the following program: Step I – 1 cycle, 20 seconds at 95ºC and Step II – 40-50 cycles, 3 seconds at 95ºC and 30 seconds at 60ºC. Finally, results were analysed by calculating the relative mRNA levels using the adapted formula (2.2), which infers the number of messenger RNA (mRNA) molecules of the interest gene per 1000 molecules of the endogenous controls[97]. GraphPad Prism 7 Software was also used.

$$(2.2)\ \text{Relative mRNA level} = 2^{-\Delta Ct} \times 1000$$

2.8. Cell proliferation measurement

To study cells proliferative capacity CellTrace™ CFSE cell Proliferation Kit (Invitrogen™, Life Technologies) was used. Immediately before use, 5 µM CellTrace™ Carboxyfluorescein succinimidyl ester (CFSE) stock solution was prepared by adding 18 µL of provided DMSO to one vial of CellTrace™ CFSE dye and mixing. Cells were resuspended in prewarmed 0.1% BSA-PBS at a final concentration of $1x10^6$ cells/mL and incubated with 2 µL of 5 µM CellTrace™ CFSE stock solution per mL for 20 minutes at 37ºC. 5 volumes of ice-culture medium were added, and cells incubated for 5 minutes on ice. Cells were pelleted by centrifugation at 200g for 5 minutes and washed 3 times by resuspending in warmed media. After the last wash, cells were resuspended in the desired volume of fresh medium and divided by wells. Cells were cultured for 5 days. Samples were collected every 24 hours and cell fluorescence was measured using Attune® Acoustic Focusing Cytometer. Cell proliferation index was determined using FlowJo V.10 and ModFit LT3.2 software. The parental generation was set based on the analysis of data from the cells maintained at 24 hours in culture after CFSE staining.

2.9. Wound-healing migration assay

Cells were cultured in a 12-well plate until confluence and then a uniform scratch was made using a 200 µL pipette tip. The suspended cells were washed, and medium was changed. The wound was imaged every 24 hours with an inverted microscope equipped with a digital camera[98]. For analysis, a fixed area of the wound was defined and measured using ImageJ software. Every 24 hours, the area occupied by migratory cells was measured and discounted to the initial area until all the wound was filled by cells.

3. Results

3.1. E-SL and sLe$^{x/a}$ antigens are expressed in colon and breast cancer tissues

Aberrant expression of sLe$^{x/a}$ by cancer cells is associated with a higher propensity for cancer progression and metastasis[70]. Using E-Ig chimera in paraffin-embedded cancer tissue sections, namely, colon adenocarcinoma and Triple Negative Breast Cancer (TNBC) tissues, we started by evaluating the expression of E-SL, whose sLe$^{x/a}$ is the main prototype[65]. Figure 3.1 shows that cancer tissues express E-SL on the apical membranes of cancer cells as well as on the goblet cells also in its apical pole (Figure 3.1 G and H). In the control assays, where the protocol was performed without E-Ig chimera or in the presence of EDTA, a calcium chelator, staining was not detected (Figure 3.1 B-C and E-F). We also observed that E-Ig chimera staining in TNBC tissues, was weaker (Score = 6) when compared to colon cancer tissues (Score = 9).

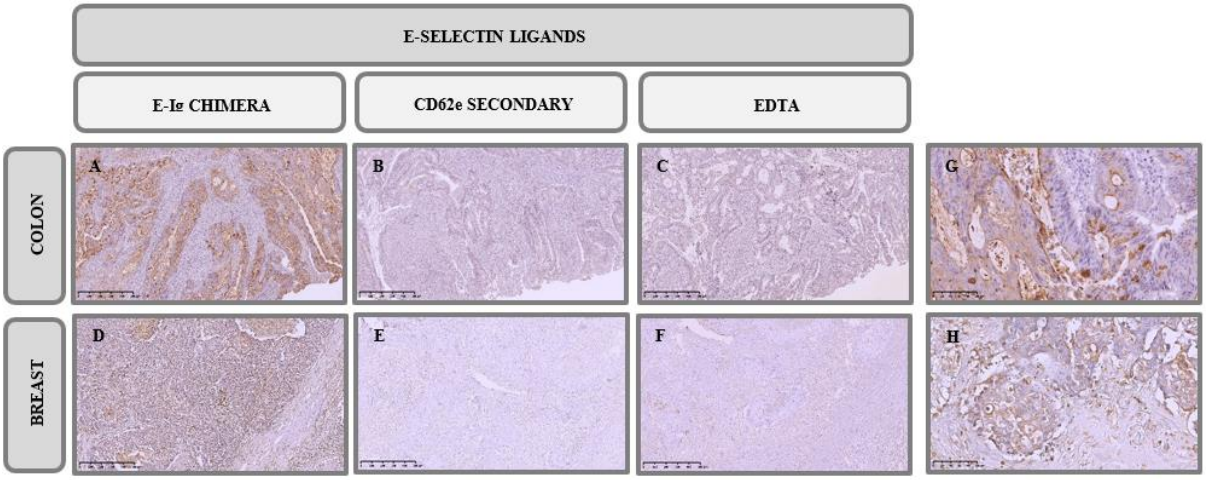

Figure 3.1 – Immunohistochemistry analysis of E-SL expression in cancer tissues. IHC staining of **A.** colon adenocarcinoma and **D.** TNBC tissues with E-Ig chimera (100X). For control, staining was performed **B. E.** in the absence of E-Ig and **C. F.** in the presence of EDTA, a calcium chelant (100X). In **G.** and **H.** is represented the same as in A. and D. but with higher magnification (400X). Brown colour indicates E-Ig positive reactivity. From A. to F. scale bar represents 500 µm while in G. and H. represents 100 µm.

The expression of sLe$^{x/a}$ was also detected in colon and breast cancer tissues using three different antibodies, HECA-452 mAb (Figure 3.2 A and D), anti-CD15s mAb (Figure 3.2 B and E) and anti-CA19.9 mAb (Figure 3.2 C and F), whose recognized epitopes are sLe$^{x/a}$, sLe^{x} and sLe^{a} respectively. In this case, we observed that although the apical membranes were stained, some weaker staining was detected on the lamina propria, except in TNBC tissues where anti-CA19.9 mAb did not stained (Score = 0). The staining with CD15s and CA19.9 mAbs complement the staining of HECA-452 mAb, however, in colon cancer tissues the expression of sLe^{x} seems to be more significant (Score = 6) than the expression of sLe^{a} (Score = 3). With both antibodies we can observe some staining associated to the secretory pathways, even more, we also notice some mucus staining. Once again, we observed that sLe$^{x/a}$ staining in TNBC tissues, was weaker (Score = 2) when compared to colon cancer tissues (Score = 8). We can confirm that the expression of sLe$^{x/a}$ is closely associated with the expression of E-SL.

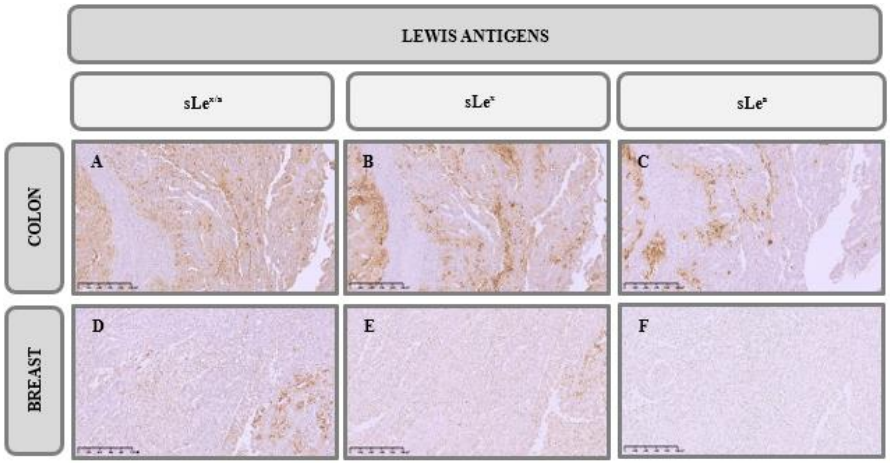

Figure 3.2 – Immunohistochemistry analysis of sLe$^{x/a}$ expression in cancer tissues. IHC staining of colon adenocarcinoma tissues with **A.** HECA-452 mAb, **B.** anti-CD15s mAb and **C.** anti-CA19.9 mAb, and TNBC tissues with **D.** HECA-452 mAb, **E.** anti-CD15s mAb and **F.** anti-CA19.9 mAb (100X). Brown colour indicates antibodies positive reactivity. Scale bar represents 500 μm.

3.2. Colorectal and breast cancer tissues analysis for relevant biomarkers

After identifying the expression of E-SL, and in particular sLe$^{x/a}$, we have attempted to characterize some biomarkers. We assessed the expression of potential scaffold proteins relevant to adhesion and signalling, namely MUC1, as well as the expression of CK, an epithelial marker, and Ki-67, a nuclear protein expressed in all stages of the cell cycle (G1, S, G2 and Mitosis) and absent in quiescent cells (G0), i.e., a proliferation marker[99].

We have found a significant staining of CK in the cytoplasm, with membrane reinforcement, in all cancer tissues (Score = 12) (Figure 3.3 A and G) confirming their epithelial origin. In fact, control tissues also showed a significant staining (Score = 8), maybe stronger, of this marker in the epithelial layers (Figure 3.3 D and J). On the other hand, the staining with MUC1 showed different profiles in different tissue types, staining weakly (Score = 2) the cytoplasm secretory pathways and the apical membrane of colon cancer tissues and was not detected in TNBC tissues (Score = 0) (Figure 3.3 B and H). In addition, we observed that normal colon tissue also express MUC1 in the epithelial barrier (Score = 3).

However, when we assessed MUC1 expression using the homemade 5E5 mAb, that recognize MUC1 GalNAc-glycosylated form, i.e., with O-linked patterns, we found that expression was much more significant in both, colon (Score = 9) and TNBC (Score = 6) tissues (Figure 3.3 C and I), whereas in the control no expression was detected (Score = 0) (Figure 3.3 F and L). Interestingly, the expression of MUC1 GalNAc-glycosylated form is diffuse in colon cancer tissues and more heterogeneous in TNBC tissues.

Finally, we evaluated the expression of Ki-67 proliferative marker, whose staining confirmed that they were proliferating tissues since a lot of nuclei stained were detected in both, colon (77%) and TNBC (59%) tissues (Figure 3.4).

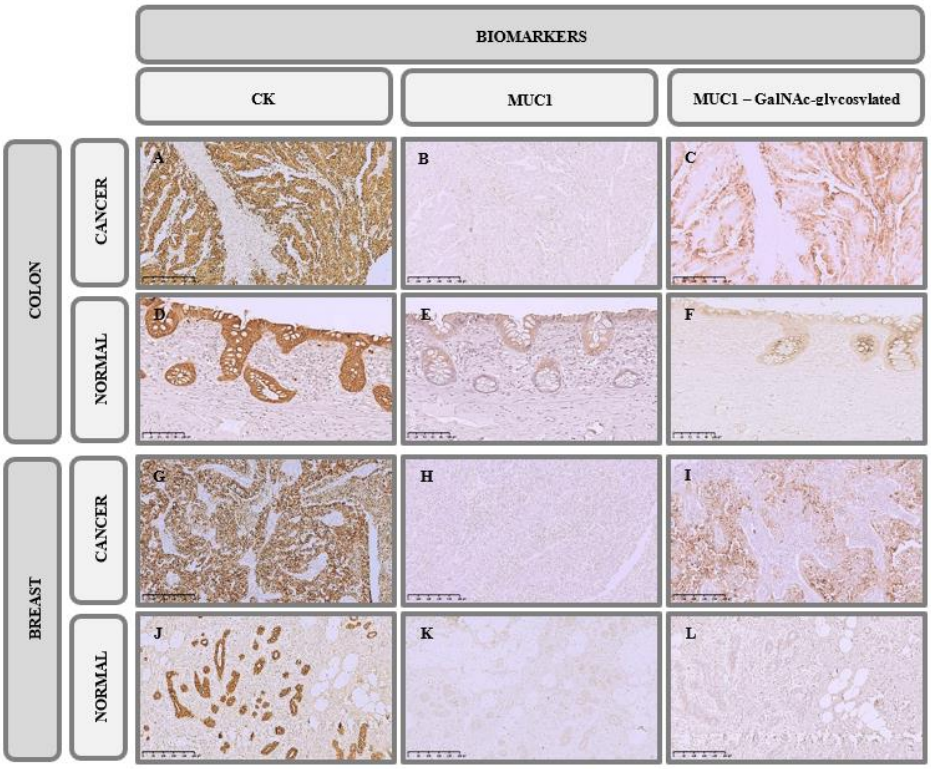

Figure 3.3 - Immunohistochemistry analysis of CK and MUC1 expression in cancer tissues. IHC staining of colon adenocarcinoma tissues with **A.** anti-CK mAb **B.** anti-MUC1 (VU4H5) mAb and **C.** anti-MUC1 (5E5) mAb, and TNBC tissues with **G.** anti-CK mAb **H.** anti-MUC1 (VU4H5) mAb and **I.** anti-MUC1 (5E5) mAb (100X). Normal colon tissue (D-F) (200X) and breast tissue (J-L) (100X) stained with the same antibodies are also represented. Brown colour indicates antibodies positive reactivity. Scale bar represents 500 μm except in D., E. and F. where it represents 250 μm.

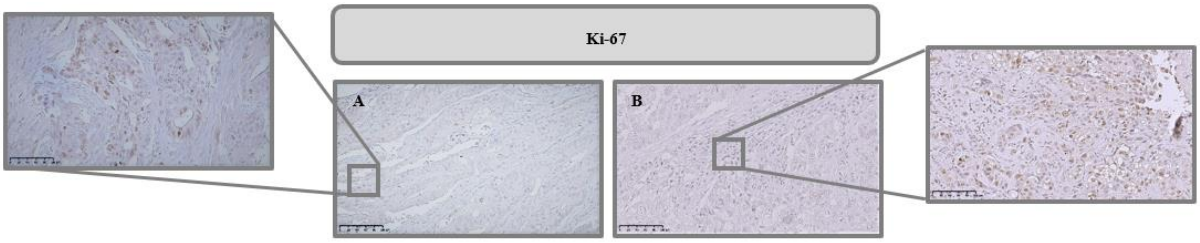

Figure 3.4 - Immunohistochemistry analysis of proliferative markers in cancer tissues. IHC staining of **A.** colon adenocarcinoma and **B.** TNBC tissues with anti-Ki-67 mAb (200X). Brown colour indicates Ki-67 positive reactivity. Scale bar represents 100 μm. Selected area with 400X magnification.

3.3. Expression of E-SL and sLe$^{x/a}$ epitopes in cancer cell lines

Considering the role of E-SL in cancer progression and metastasis[66], we started by evaluating its expression in our cellular experimental models. Expression of E-SL in cancer cell lines was assessed by flow cytometry using E-Ig chimera as a probe. As shown in Figure 3.5, HT29, LS174T and CF1T cell lines express E-SL, the expression in CF1T cells is lower compared to HT29 and LS174T cells. On the other hand, the SW48 cell line does not express E-SL at the cell membrane.

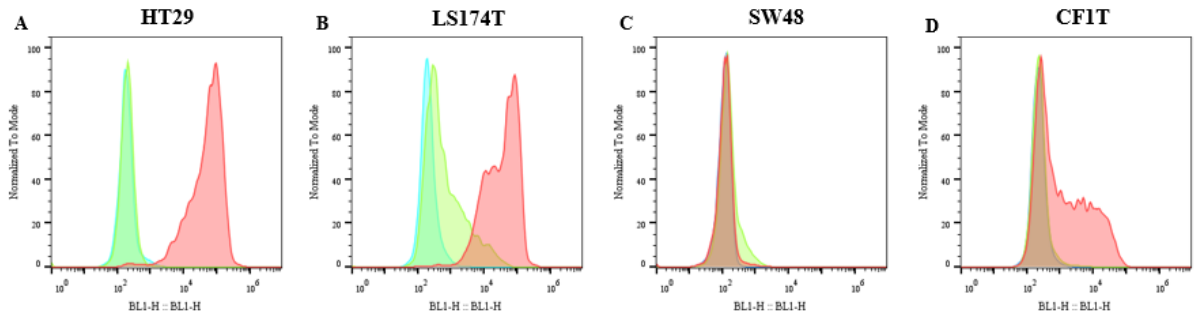

Figure 3.5 - E-SL expression in cancer cell lines. Flow cytometry analysis of E-SL expression in **A.** HT29, **B.** LS174T, **C.** SW48 and **D.** CF1T cell lines. Cancer cell lines were stained with E-selectin -Ig chimera (red). Unstained cells (blue) and EDTA (green) are also shown as a control. The xx axis represents fluorescence intensity of the fluorophore and yy axis represents the normalized event count.

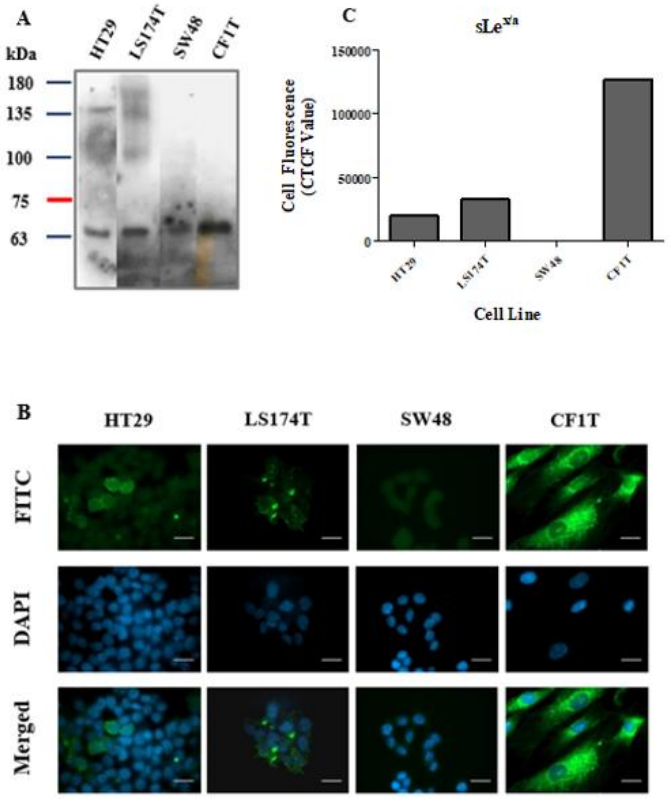

Figure 3.6 - sLe$^{x/a}$ expression and cellular distribution in cancer cell lines. A. Western blot analysis of sLe$^{x/a}$ expression in HT29, LS174T, SW48 and CF1T cell lines. Cell lysates was resolved by SDS/PAGE electrophoresis and immunoblotted with HECA-452 mAb which recognizes sLe$^{x/a}$ epitopes. kDa = kilodalton **B.** Fluorescence microscopy images of sLe$^{x/a}$ epitopes. On the first line is presented sLe$^{x/a}$ labelled with FITC antibody (green), on second line nuclei stained with DAPI (blue) and on the third line merged image. Scale bar: 1 µm. **C.** Cell fluorescence quantification.

Then, we confirm the expression of sLe$^{x/a}$ since, in addition to be the main binding motif for E-Selectin, they play an important role in the early stages of malignant transformation[100]. There is a positive correlation between aberrant expression of these glycans on the primary cancer cells and cancer

progression and metastasis[50]. By WB we found that HT29 and LS174T cell lines express $sLe^{x/a}$ whereas in SW48 and CF1T cell lines the expression was not detected (Figure 3.6 A), which is consistent with the fact that SW48 cell line doesn't express E-SL and CF1T cell line has a low expression of E-SL. Besides that, the expression pattern varies in these two cell lines, in HT29, two bands corresponding to ≈ 140 kDa and 100 kDa proteins are present, while in the LS174T a third band with ≈ 170 kDa appears.

We also assessed the distribution of $sLe^{x/a}$ in these cell lines by Immunofluorescence (IF) to identify and localize the expression of this glycan. As shown in Figure 3.6 B, $sLe^{x/a}$ is expressed essentially along the cell membrane, there is also some expression in the cytoplasm which will be related to its transport to the membrane. We also noted that the distribution of $sLe^{x/a}$ on the cell membrane is not uniform. These IF results confirm the results from WB for HT29, LS174T and SW48 cell lines regarding the expression of $sLe^{x/a}$. A different situation is observed in the CF1T cell line where the cell fluorescence intensity was significant (Figure 3.6 C), contrary to what was observed in the WB where the expression was not detectable. Since the results for $sLe^{x/a}$ expression in this cell line do not complement each other and that the expression of E-SL was low, we decided to exclude this cell line in the next experiments. In future, it will be necessary to repeat this analysis. Therefore, we selected HT29 and LS174T cell lines that have a high expression of sialofucosylated epitopes and we also selected SW48 cell line that will be used as negative control.

3.4. Treatment with 2-FF decreases $sLe^{x/a}$ and increases CK expression.

Studies using mAbs have shown that fucosylated carbohydrate chains are involved in several physiological functions and also in malignant transformation[101]. α1,3 and α1,4 FUTs have been reported as being involved in the synthesis of $sLe^{x/a}$ in cancer[102]. The 2-FF inhibitor was shown to be able to inhibit FUTs activity, which reduces fucosylated glycans, like Lewis antigens, in colorectal cancer (CRC) cells[103]. To understand how fucosylation affects cancer progression and metastasis, we started by treating the cells with 2-FF fucosylation inhibitor. It is described that this inhibitor does not affect cell viability[96]. The different cell lines were treated with 2-FF (as described in section 2) and at day 5 they were removed for analysis.

As represented in Figures 3.7 A-C, in all cell lines treated with the inhibitor. the relative mRNA expression of FUT II, FUT IV, FUT V, FUT X and FUT XI decreases compared with non-treated cells, indicating that inhibition of fucosylation was efficient. We then evaluated the expression of $sLe^{x/a}$ by flow cytometry and a decrease in their expression was observed in HT29 and LS174T cell lines (Figures 3.7 D-G). As expected, although the inhibition of FUTs occurred, no effect was observed at $sLe^{x/a}$ expression in SW48 cell line (Figures 3.7 E-F). Thus, $sLe^{x/a}$ is a fucosylated epitope, i.e., the inhibition of fucsoylation leads to loss of its expression as a result of decreased expression of FUTs.

$sLe^{x/a}$ is the minimal recognition motif for E-selectin which is involved in tumour cells addition to the endothelium before they invade new tissues[64], this process evolves structural and cellular morphology modifications, so, we evaluated the effect of fucosylation, and consequent decrease in $sLe^{x/a}$ expression, in the cytoskeleton, namely in CK components, that are involved in important functions like cytoplasm organization, transport and cell division[104]. Furthermore, they have been described as very useful markers in epithelial malignancies[105].

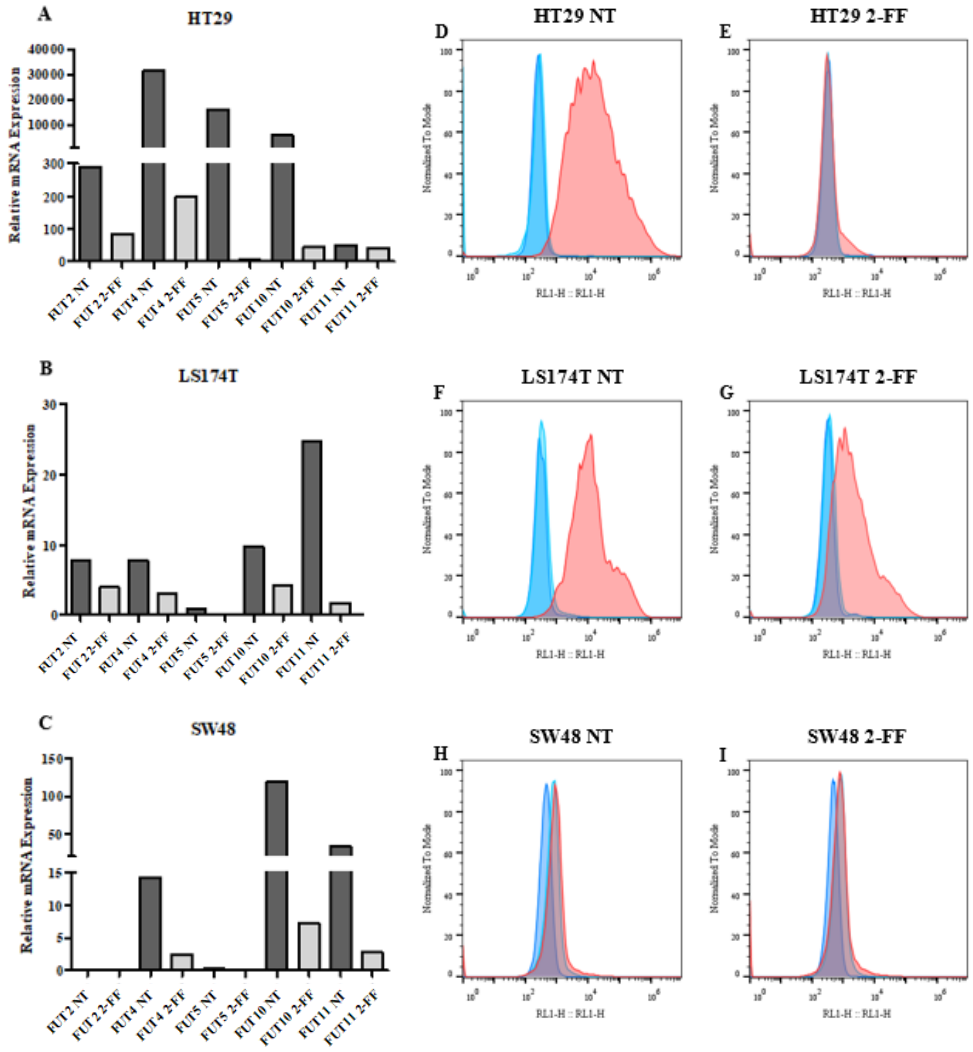

Figure 3.7 - Effect of fucosylation inhibitor in sLe$^{x/a}$ expression. A-C. RT-qPCR analysis of FUTs gene expression in **A.** HT29, **B.** LS174T and **C.** SW48 cell lines treated with 2-FF inhibitor (2-FF) or not (NT) for 5 days. Values correspond to the amount of mRNA copies of each FUT per 1000 mRNA copies of β-actin and GAPDH housekeeping genes. NT = not treated. **D-I.** Flow cytometry analysis of sLe$^{x/a}$ expression after 2-FF treatment. In **D.** HT29 **F.** LS174T and **H.** SW48 cell lines were not treated and in **E.** HT29, **G.** LS174T and **I.** SW48 cells were treated with 2-FF inhibitor. Cancer cell lines were stained with HECA-452 mAb (red) or only with secondary antibody (light blue). Unstained cells (dark blue) are also shown. The xx axis represents fluorescence intensity of the fluorophore and yy axis represents the normalized event count.

Cells from different cell lines were treated with 2-FF or not and evaluated for CK expression. By flow cytometry, we assessed that in HT29 and LS174T cell lines treated with the fucosylation inhibitor, the percentage of cells expressing CK increase compared to not-treated cells (Figure 3.8 A-D), suggesting that a decrease in sLe$^{x/a}$ expression may lead to changes in CK expression. On the other hand, in SW48 cell line, where the inhibition of fucosylation didn't produce effects at sLe$^{x/a}$ expression, no differences in CK expression were observed (Figure 3.8 E-F). Considering this increase, we assessed the expression pattern of CK in each cell line using a pan-CK mAb. WB analysis revealed that inhibition of fucosylation not only leads to increased expression of CK, but also that the type of CK whose expression is increased varies depending on the cell line, which was revealed by the appearance of two bands with ≈ 45 and 51 kDa in HT29 cell line and one band of ≈ 40 kDa in LS174T cell line (Figure 3.8 G). According to the results of flow cytometry, in SW48 cell line there is no difference between treated with 2-FF and not-

treated cells (Figure 3.8 G). Changes in cell adhesion, caused by decreased expression of $sLe^{x/a}$, besides increasing the expression of proteins that compose the intermediate filaments of the cytoskeleton, seems to induce different profiles of expression in different cell lines.

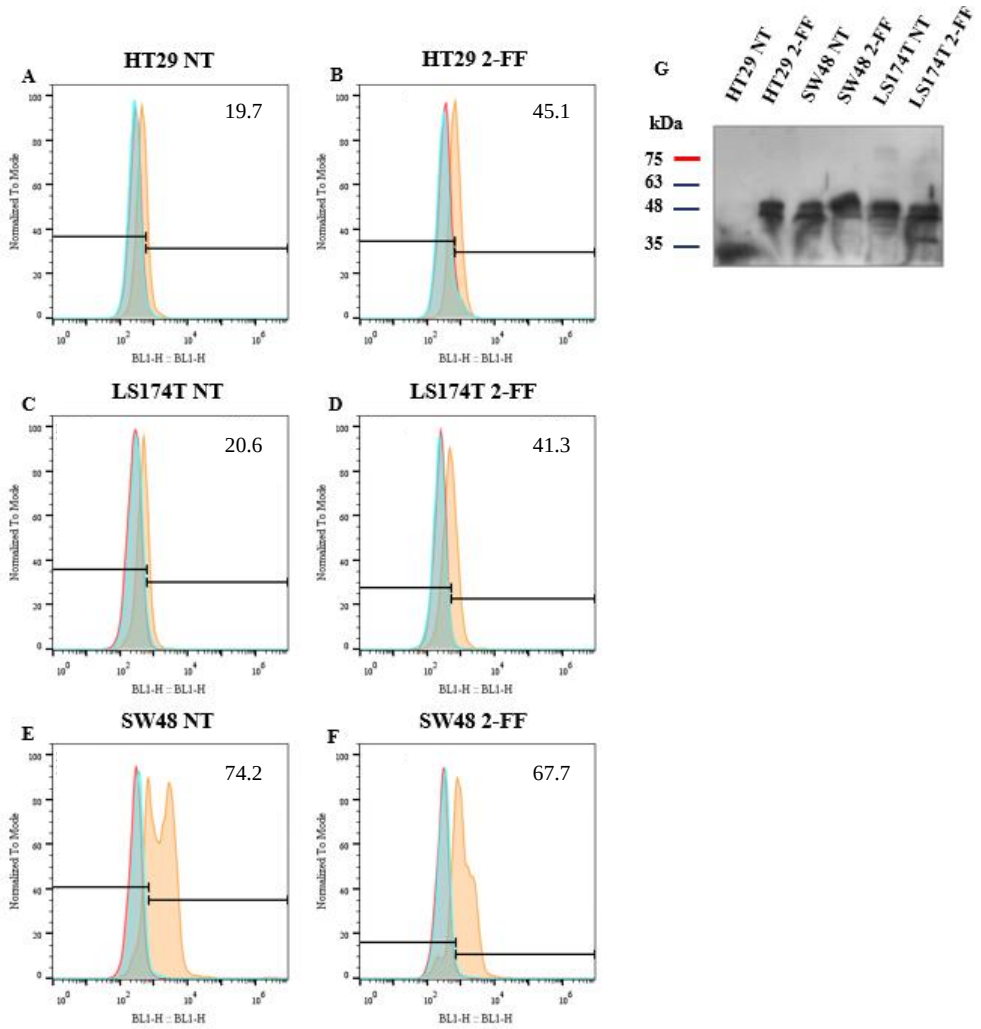

Figure 3.8 - Effect of fucosylation inhibitor in CK expression. A-F. Flow cytometry analysis of CK expression after 2-FF treatment. In **A.** HT29 **C.** LS174T and **E.** SW48 cell lines were not treated and in **B.** HT29, **D.** LS174T and **F.** SW48 cells were treated with 2-FF inhibitor. Cancer cell lines were stained with pan-CK (AE1/AE3) mAb (orange) or only with secondary antibody (light blue). Unstained cells (red) are also shown. The xx axis represents fluorescence intensity of the fluorophore and yy axis represents the normalized event count. **G.** WB analysis of CK expression in HT29, LS174T and SW48 cell lines. Cell lysates was resolved by SDS/PAGE electrophoresis and immunoblotted with pan-CK (AE1/AE3) mAb which recognizes type I and type II CK. NT = not treated. kDa = kilodalton

3.5. MUC1 is a potential scaffold protein in initiating cancer progression and metastases

As previously mentioned, the scaffold proteins decorated with a specific glycan play an important role in E-selectin recognition. Since mucins, and in particularly MUC1, have been implicated in E-selectin binding[78] and in increasing the metastatic potential of tumour cells[80], we evaluate the expression of MUC1 in cancer cell lines. As shown in Figure 3.9 A-F, the expression of MUC1 was not detected in

all cell lines by flow cytometry. During tumour development, changes in MUC1 expression are responsible for the loss of cell polarity[76], so, it is possible that MUC1 change your location. To corroborate this hypothesis, we performed an IF assay where we permeabilize the cells to access the cytoplasm. We found that all cell lines have expression of MUC1 glycoprotein in cytosol, probably associated with cellular transport pathways (Figure 3.9 G). Furthermore, the treatment with 2-FF in HT29 and LS174T cell lines led to an increase in fluorescence associated with MUC1 expression (Figure 3.9 H), which seems to be consistent with studies showing that a decrease of glycosylation may lead to transmembrane MUC1 uptake[106], i.e., changes in its location and consequent loss of polarity. Regarding the SW48 cell line, our negative control, inhibition of fucosylation had no effect on MUC1 expression (Figure 3.9 H), suggesting that fucosylation may play an important role in the transport of MUC1 to the membrane.

Some studies show that MUC1 may be involved in the suppression of the inflammatory response mediated by T cells in cancer[107,108]. We sought to evaluate if these cell lines were able to secrete MUC1 into the culture medium, where it could behave as an inhibitory factor. Figure 3.9 I shows the WB of culture media from the different cell lines, collected over the time, where secreted MUC1 form was not detected.

Therefore, in these cell lines, MUC1 expression is detected in the cytoplasm and fucosylation inhibitors leads to increased fluorescence signal as a result of the MUC1 uptake.

3.6. Inhibition of fucosylation decreases cell proliferation and migratory capacity

It has been reported that suppression of FUTs expression is able to attenuate cell proliferation in cancer[108,109]. We assessed the effect of fucosylation on proliferation using the CFSE dilution method to measure proliferation indexes in cell lines. We observed that HT29 and LS174T cell lines treated with 2-FF inhibitor show lower proliferation rates than not-treated cells, which results in less accelerated proliferation (Figures 3.10 A-D and G). In SW48 cell line, although the inhibition of fucosylation has some effect on cell proliferation, its proliferative capacity is less pronounced than in the remaining cell lines (Figures 3.10 C-D and G), which consolidate the influence of $sLe^{x/a}$ on proliferation induction in cancer cells.

Since we have observed that the decrease in E-SL expression, namely $sLe^{x/a}$, as a result of the treatment with 2-FF inhibitor, is implicated in increasing CK expression, and that the role of E-SL, also in increasing the cellular metastatic potential of tumour cells, has been studied[66], we assessed the influence that fucosylation will have on migration in cancer cell lines through a wound-healing assay. We verified that, in all cell lines, cells treated with 2-FF inhibitor take longer to fill the space left by the scratch, i.e., they had a lower capacity to migrate compared to not treated cells (Figure 3.10 H). These results show that inhibition of fucosylation contributes to reduce proliferation rates and cell migration ability.

3.7. Fucosylation increases growth factors and proinflammatory cytokines expression in cancer cells

Since increased proliferation and cell migration is related to the expression of growth factors and proinflammatory cytokines that affect certain signalling pathways[110], we evaluated the effect of inhibition of fucosylation on the expression of some growth factors and proinflammatory signals.

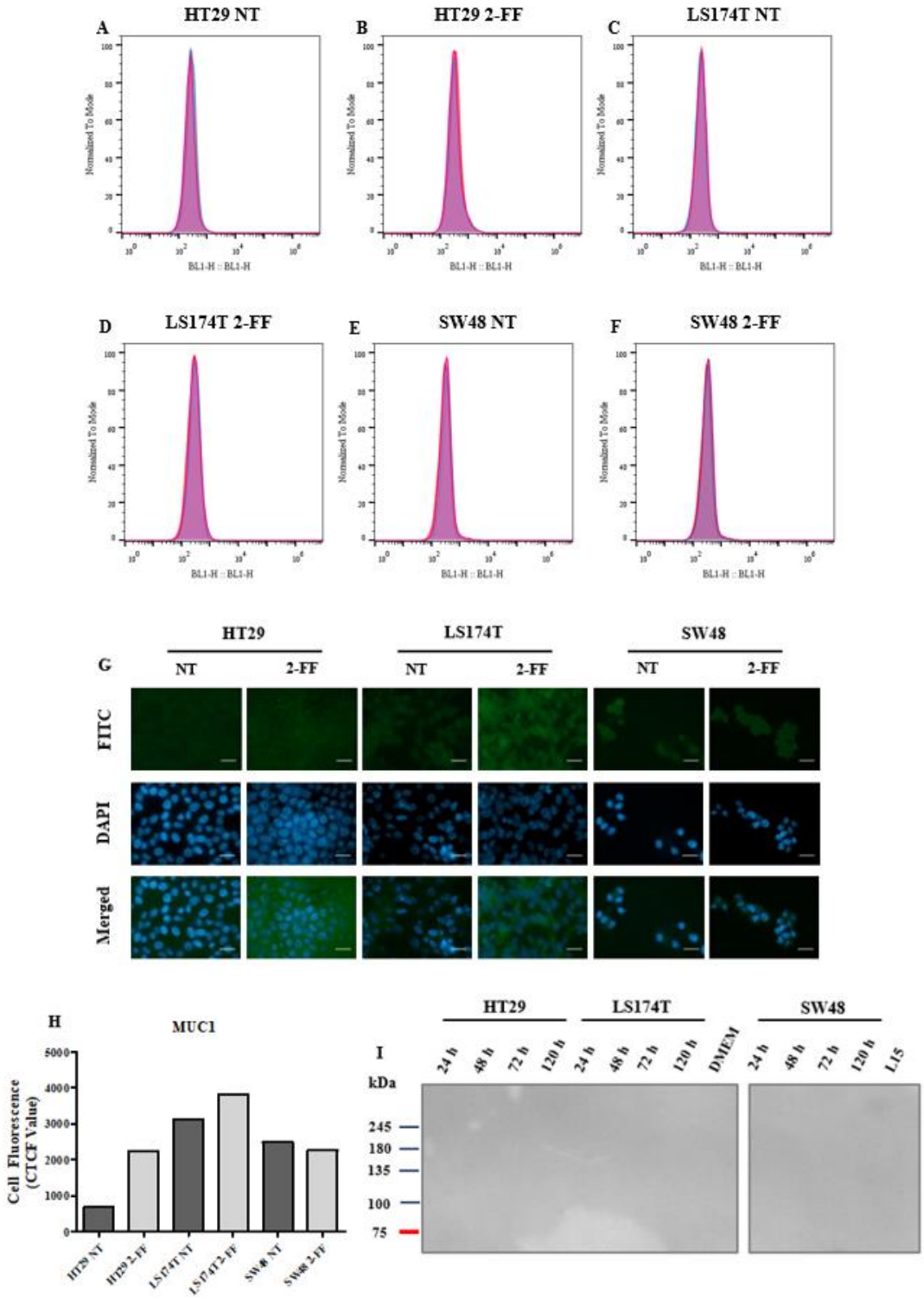

Figure 3.9 - MUC1 expression analysis in cancer cell lines. A-F. Flow cytometry analysis of MUC1 expression after 2-FF treatment. In **A.** HT29 **C.** LS174T and **E.** SW48 cell lines were not treated and in **B.** HT29, **D.** LS174T and **F.** SW48 cells were treated with 2-FF inhibitor. Cancer cell lines were stained with anti-MUC1 (VU4H5) mAb (pink) or only with secondary antibody (light blue). Unstained cells (red) are also shown. The xx axis represents fluorescence intensity of the fluorophore and yy axis represents the normalized event count. **G.** Fluorescence microscopy images of MUC1. On the first line is presented MUC1 labelled with FITC antibody, on second line nuclei stained with DAPI (blue) and on the third line merged image. Scale bar: 1 μm. NT = not treated. **H.** Cell fluorescence quantification. **I.** WB analysis of secreted MUC1 in HT29, LS174T and SW48 cell lines. The supernatants of all cell lines at 24 h, 48 h, 72 h and 120 h were resolved by SDS/PAGE electrophoresis and immunoblotted with anti-MUC1 (VU4H5). Not supplemented DMEM and L15 cultures medium were used as control. h = hours. kDa = kilodalton

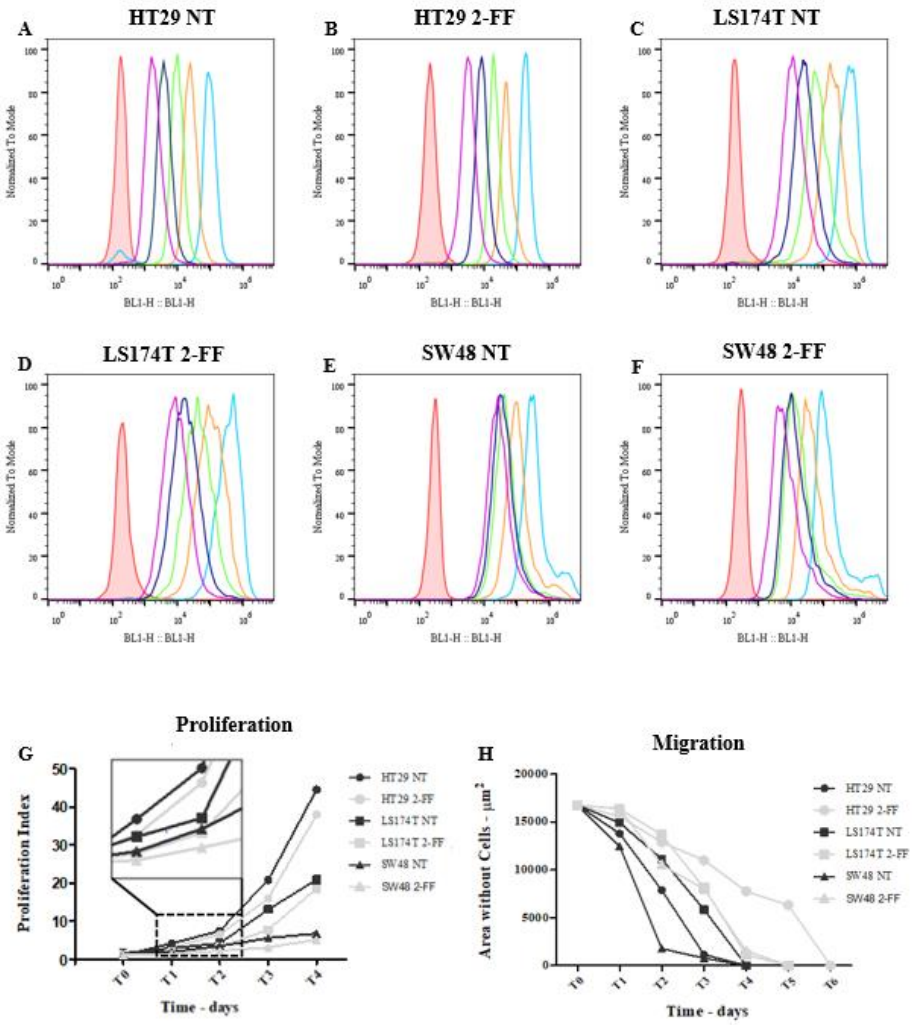

Figure 3.10 - Inhibition of fucosylation reduced cell proliferation rates and cell migration capacity. A-F. Effect of 2-FF treatment on cell lines proliferation determined by CFSE dilution method and analysed by flow cytometry. In **A.** HT29 **C.** LS174T and **E.** SW48 cell lines were not treated and in **B.** HT29, **D.** LS174T and **F.** SW48 cells were treated with 2-FF inhibitor. Cells were in culture during 24 h (light blue), 48 h (orange), 72 h (green), 96 h (purple) and 120 h (pink) and then analysed. Unstained cells (red filled curve) are also shown. The xx axis represents fluorescence intensity of the fluorophore and yy axis represents the normalized event count. **G.** Cancer cell lines proliferation indexes. **H.** Cancer cell lines migration capacity. NT = not treated

In all cell lines, in cells treated with 2-FF inhibitor there is a decrease in the expression of TGF-β, Fibroblast Growth Factor (FGF2) and Vascular Endothelial Growth Factor A (VEGF-A) growth factors compared to not treated cells (Figures 3.11 A-C). Regarding the expression of IL-1β and IL-6, the proinflammatory cytokines, we verified different responses to the inhibition. In LS174T and SW48 cell lines there is a decrease in the expression of proinflammatory cytokines when the inhibition of fucosylation is done, whereas in the HT29 cell line the treatment with 2-FF led to an increase in the markers of inflammation (Figures 3.11 D-E). This result is consistent with some studies that show a duality in the function of the IL-1β cytokine[111] and consequent modulation of the inflammatory context.

Thus, inhibition of fucosylation affects the expression of certain growth factors, with importance in the tumour microenvironment, and is able of modulating the expression of proinflammatory cytokines which may depend on the type of cell line subject to the treatment.

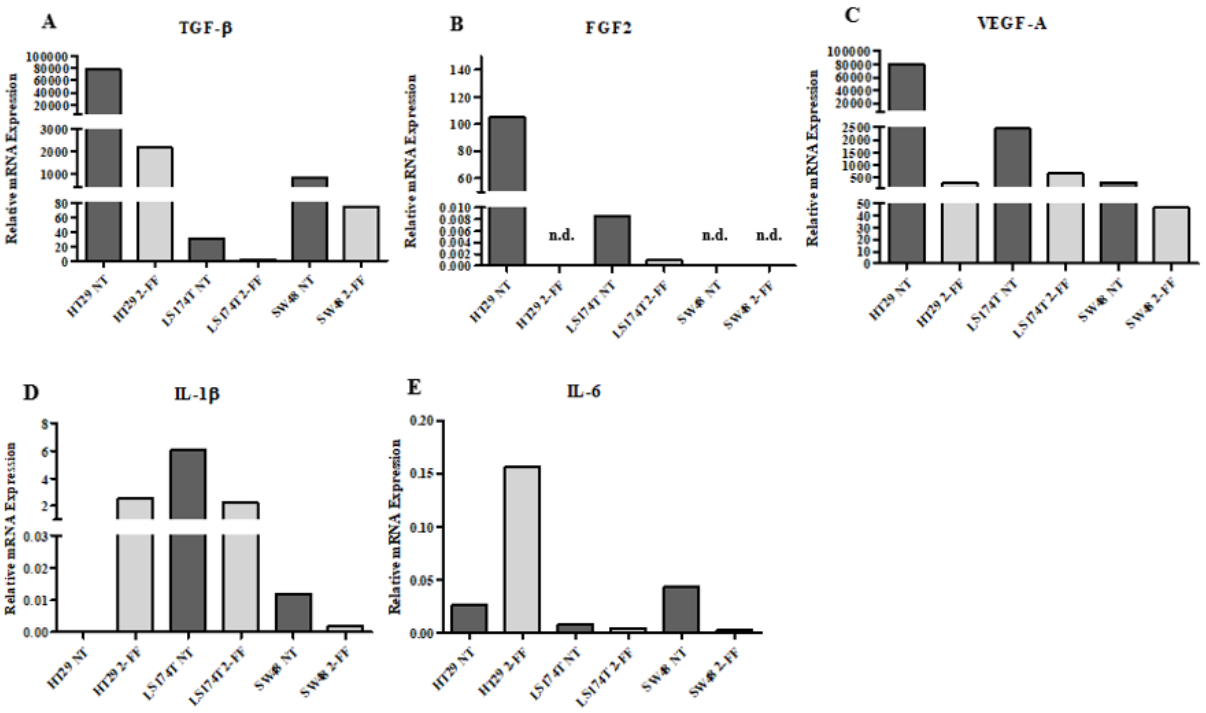

Figure 3.11 - Growth factors and proinflammatory cytokines expression in cancer cell lines. RT-qPCR analysis of **A.** TGF-β, **B.** FGF2 and **C.** VEGF-A growth factors and **D.** IL-1β and **E.** IL-6 proinflammatory cytokines gene expression in HT29, LS174T and SW48 cell lines treated with 2-FF inhibitor (2-FF) or not (NT). Values correspond to the amount of mRNA copies of each marker per 1000 mRNA copies of β-actin and GAPDH housekeeping genes. NT = not treated. n.d. = not detected

3.8. Fucosylation plays an important role in EMT

We have shown that inhibition of fucosylation, using 2-FF inhibitor, is responsible for decreasing the expression of $sLe^{x/a}$, which can bind to E-selectin, and which in turn, appears to produce effects in the cytoskeleton, increasing CK expression. We have also shown that this process influences the cancer cell proliferation and migration.

Since EMT is an essential step in metastasis and that changes in the expression of epithelial and mesenchymal markers are associated with entry of cells in this program[85], we evaluate how fucosylation may influence EMT by assessing the expression of fibronectin in cells subjected to treatment with 2-FF inhibitor. Fibronectin has been reported as a promoter of tumour invasion and migration[90]. For this assay, we recovered CF1T cell line which was subjected to the same treatment with the fucosylation inhibitor. Figure 3.12 shows the immunofluorescence results of the cell lines where we can observe that all cancer cell lines, except the SW48, have a high expression of fibronectin. In addition, treatment with the fucosylation inhibitor led to the decreased fibronectin expression in all cell lines. This effect is more significant in CF1T and LS174T cell lines. Thus, fucosylation seems to condition the expression of this ECM protein.

Then, we assessed the expression of CK and we found that, in all cell lines, the same treatment increased the expression of CK that, initially, were poorly expressed. Taken together, our observations suggest that fucosylation plays an important role in the entry of cells into EMT program, characterized by increased expression of mesenchymal markers accompanied by decreased epithelial marks expression.

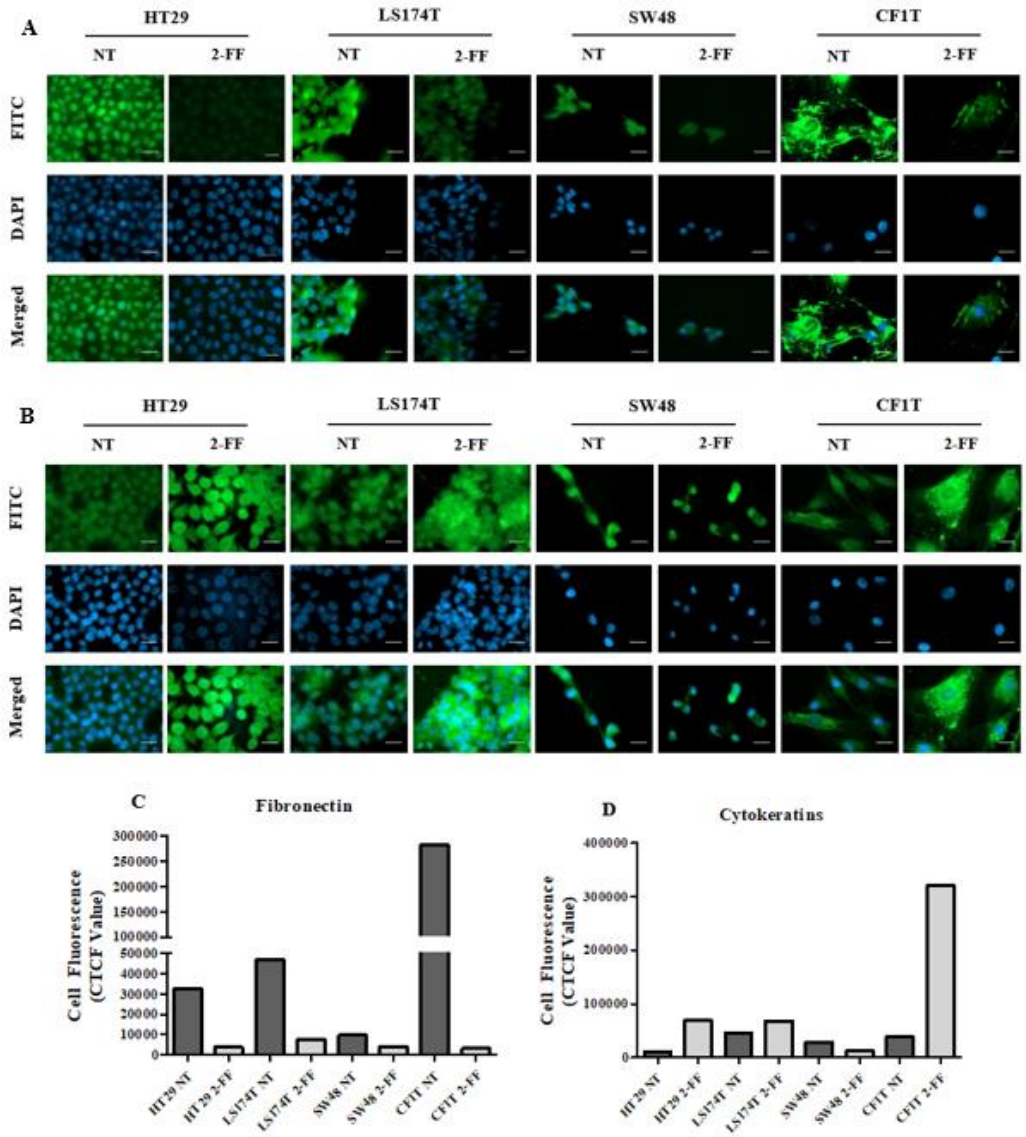

Figure 3.12 - Inhibition of fucosylation reverses the expression of EMT markers. Fluorescence microscopy images of **A.** fibronectin and **B.** CK. On the first line is presented fibronectin or CK labelled with FITC antibody, on second line nuclei stained with DAPI (blue) and on the third line merged image. Scale bar: 1 μm. NT = not treated. In **C.** and **D.** are represented the cell fluorescence quantification of fibronectin and CK, respectively.

4. Discussion

Glycosylation is a fundamental process in malignant transformation[47,112]. Changes in glycosylation patterns have an impact, not only in cancer cell growth and survival, but also appears to be involved in invasion and metastasis[44]. In fact, cancer cell aberrant glycosylation is now considered to be a universal Hallmark of cancer[47].

In general, increased expression of $sLe^{x/a}$, two of the main fucosylated antigens, as well as FUTs, is reported in different types of cancer, like colon[113], breast[56] and lung cancer[57], has been closely associated with cancer progression and potentially correlated with metastasis, the leading cause of death in cancer[41].

In this thesis, we had as main objectives to evaluate the expression of aberrant glycosylation patterns in cancer, especially the expression of $sLe^{x/a}$, to identify new biomarkers as potential therapeutic targets, namely candidates to scaffold proteins decorated with certain glycosylation patterns, and understand the role of these glycans, as well as fucosylation, in cancer progression and metastasis.

Considering the role of E-selectin and E-SL, of which $sLe^{x/a}$ is the main binding motif, in the initial steps of tissue invasion and metastasis, we started by characterizing the expression of these markers in tumour tissues by Immunohistochemical staining of colon adenocarcinoma and TNBC tissue sections. We have found that both tissues express E-SL, essentially in the plasma membrane, and the staining was more intense in colon tissues than in breast tissues. We also noticed the calcium dependence of ligands binding to E-selectin, in fact, when the staining protocol was developed in the presence of a calcium chelator, such as EDTA, the staining is no longer detected, and we obtain a result similar to the staining without E-Ig. Then, we proceeded to access the expression of $sLe^{x/a}$ using three different antibodies. The staining patterns were similar to E-Ig staining, but with positive reactivity on the lamina propria in addition to staining associated with the secretory pathways. We have found that anti-CD15s mAb and anti-CA19.9 mAb staining complement the staining of HECA-452 mAb, however, discrimination of each marker allowed us to conclude that in colon tissues the sLe^{x} expression is stronger than sLe^{a} expression. These results are consistent with the literature where it is reported the role of E-selectin in cancer progression[66] and the importance of its ligands as biomarkers that allow a better characterization of each type of tumour[67,114]. In fact, the CA19.9 marker is currently used in clinic as a biomarker in several types of cancer, such as pancreatic and colon cancer[115].

Confirmed the expression of E-SL and $sLe^{x/a}$, we characterized tumour tissues regarding the expression of some biomarkers that could potentially be useful in the study of cancer progression, namely the expression of CK, which are part of cytoskeleton and obligatorily involved in structural and morphological changes related to migration, as well as the expression of MUC1 which be associated with epithelia, can be a potential scaffold protein candidate for aberrant glycosylation patterns and also play a role in cell adhesion and tumour signalling[80]. In fact, we confirmed that all tissues expressed CK in the cytoplasm of cells in the epithelial layer. This result is obviously related to the epithelial origin of the tissue samples analysed. Regarding MUC1, our results showed that staining of MUC1 GalNAc-glycosylated form was more intense than the non-glycosylated form detected only in colon cancer tissues. However, in normal tissues the expression of non-glycosylated MUC1 in the colon was detected.

The staining is positive essentially on the membrane and some staining was also detected in the secretory pathways. These results suggest that MUC1 is a potential scaffold protein for the aberrant glycosylation pattern acquired during tumour development since it is present in cancer and already has an O-glycosylated pattern, however, remember that in the same glycoprotein more than one glycosylation pattern can be present. Moreover, the glycosylation pattern carried by MUC1 may consist of Lewis antigens[78]. If we look at E-Ig chimera staining, as well as HECA-452 mAb, mucus was also detected in

some areas. Mucus is mainly composed by mucins and there are already some studies reporting the secretion of MUC1 in malignant context[116]. Further assays will have to be developed to characterize the role of MUC1 in cancer progression, based on its glycosylation pattern.

Still considering tissues, its proliferative state was confirmed by Ki-67 staining. In fact, both colon and breast cancer tissues have high percentages of proliferation, which is related to a higher cancer progression and consequently with a higher invasion capacity, being therefore more aggressive tumours. Ki-67 is also a biomarker with clinical importance[117].

Thus, we can conclude that colon adenocarcinoma and TNBC tissues, both of epithelial origin, express E-SL, including $sLe^{x/a}$, in the apical membranes of cancer cells, although in different amounts. We may also conclude that MUC1 appears to play a role in tumour development, possibly associated with tumour signalling since some of its staining is in the membrane and in secretory pathways.

Considering our results obtained in tissue analysis, we attempt to study the role of sialofucosylated antigens by evaluating the effect of fucosylation inhibition on cancer progression. Using cell lines as experimental models, we started by characterizing them for the expression of E-SL and $sLe^{x/a}$, as we did in tissues. Our results confirmed that each cell type shows its own identity which concerns glycan expression as well as the expression patterns of these glycans. In fact, HT29, LS174T and CF1T cell lines showed E-SL expression, whereas in SW48 cell line the expression was not detected. When we accessed the expression of $sLe^{x/a}$, the results were concordant, in fact, $sLe^{x/a}$ is the main binding motif to E-selectin, which explains its absence on SW48 cell line since it does not express E-SL. Next, we evaluated the $sLe^{x/a}$ expression distribution and found that it is expressed mostly along the membrane and in the cytosol, probably associated with transport and secretion pathways. Actually, the glycosylation process occurs along the ER-*cis/trans* Golgi network and proteins are then transported to the membrane[16]. In this case, we can confirm that $sLe^{x/a}$ is being expressed and carried to the membrane where it plays a part of its functions, which would be expected since E-SL are transmembrane glycoproteins, most of that decorated with Lewis antigens[65]. It should be noted that $sLe^{x/a}$ location along the membrane is not uniform, this observation is related to loss of polarity associated with changes during malignant transformation. Interestingly, these results corroborate our analysis of these markers in tumour tissues.

The CF1T cell line did not produce such consistent results since the expression of $sLe^{x/a}$ was no detected by WB in contrast to IF assays where the expression seems to concentrate in the cytoplasm. These results may be related to the sensibility of the techniques, in the future, it will be necessary to re-evaluate this situation.

Fucosylation is involved in several processes, including malignant transformation[44]. To evaluate the role of fucosylation in cancer progression and metastasis, we have studied the effect of inhibition of fucosylation on cancer cell lines. We selected the HT29 and LS174T cell lines by expressing high amounts of E-SL and $sLe^{x/a}$, SW48 cell line was also maintained as negative control. Then, we started by treating them with the 2-FF fucosylation inhibitor. Treatment with this inhibitor let do decreased $sLe^{x/a}$ expression at the cell surface which was confirmed by a decrease in the relative expression of FUTs mRNAs as well as the expression of their products, $sLe^{x/a}$. So, $sLe^{x/a}$ is a fucosylated epitope whose expression is dependent on the activity of FUTs. In fact, α1,3 FUTs, such as FUT IV, FUT V, FUT X and FUT XI, but especially FUT IV and FUT V, are involved in sLe^{x} expression[118]. FUT XI is quite involved in protein folding, i.e., their inhibition will also compromise the correct processing of glycans.

If we consider that $sLe^{x/a}$ is the main binding motif to E-selectin which is involved in the early stages of tissue invasion by circulating tumour cells, which in turn involves changes in cell morphology, it is likely that we verified changes in the expression of cytoskeleton components. Thus, we have accessed the effect of inhibition of fucosylation on the expression of structural proteins, such as CK, which play important roles, for example, in the transport and maintenance of cell epithelial features. In fact, inhibition of fucosylation led to an increase in CK expression. Our results suggest that fucosylated glycans may play a role in this increased expression of CK, for example, triggering certain signalling pathways that culminate in the activation of transcription factors that target CK expression, since in SW48 cell line, where the $sLe^{x/a}$ expression was absent, CK expression remained similar. In addition, fucosylation appears to affect different CK in a different way. If we observe, cell lines subject to the same 2-FF treatment have different CK expression patterns. The most significant difference is on HT29 cell line where inhibition appears to significantly increase the expression of CK of higher molecular weights, such as basal CK, and to decrease the expression of lower molecular weights types. In LS174T cell line, we also observed differences, namely the appearance of bands corresponding to CK of lower molecular weights. In this case, the difference is not so significant maybe because of the time needed for CK recycling. It should be noted that on SW48 cell line, we did not find differences in the expression patterns. These results are consistent with studies showing the value of CK as biomarkers of tumour stages[105,119,120], further, these results confirm that in fact cancer cells are extremely heterogenous which allows to use certain expression patterns, well characterized, to identify tumour types and stages.

So, we suggested that the recognition of $sLe^{x/a}$ can induce changes in cellular structures that are part of the cancer progression, however, the glycoproteins that are decorated with these glycans also play important roles in this type of recognition and signalling[121]. Incorrect protein folding can be sufficient to compromise the functions or the expression patterns of certain products, in the same way, certain glycosylation patterns determine the protein folding. Actually, a lot of proteins decorated with $sLe^{x/a}$ are described as implicated in several steps of cancer progression[122,123]. In this context, MUC1 appears as a potential scaffold of such glycosylation patterns since it is a highly glycosylated transmembrane protein[78]. In addition, it is an epithelial protein with important functions in protection and signalling[80].

In our results, MUC1 expression at the cell surface was not detected which may be related to antibody access to its epitope. In fact, although MUC1 is overexpressed in cancer, it is mostly underglycosylated[124], furthermore, it is mostly O-linked glycosylation patterns characterized by incomplete and truncated structures, such as Tn and STn[125]. Remember that, in cancer tissues, the more intense staining was detected using the antibody against GalNAc-glycosylated form. Therefore, structural changes may hamper the recognition of Variable Number of Tandem Repeats (VNTR) by the antibody that we used. However, when we performed the IF assay to evaluate possible changes in MUC1 location, which cause loss of polarity, some expression was detected. This observation can be related to the permeabilization once we have access to the cytoplasm where proteins is being processed and transported. In addition, the protein folding during the transport to the membrane is naturally different from the final protein structure once anchored, which can make available some epitopes. Moreover, in cells treated with fucosylation inhibitor we can observe an increase of MUC1 staining in the cytoplasm. This result suggests that fucosylation may play an important role in MUC1 transport to the membrane once we have observed its accumulation inside the cell. FUT XI is involved in folding and protein sorting along secretory pathways and in fact we have found that in cells treated with 2-FF inhibitor, the expression of FUT XI mRNA was decreased, which could have as consequence defect in MUC1 transport to the membrane with consequent accumulation in the cytosol.

Indeed, in our immunohistochemical assays, staining of MUC1 in colon cancer tissues, although weak, was detected in the cytoplasm associated with secretory pathways. These results may also be related to

other studies showing that underglycosylated MUC1 exhibits a higher internalization rate through claterin-mediated endocytosis without increasing its degradation[106], which in part results in loss of cell polarity[124]. Taken together, these data are also according to other studies that show the role of MUC1 in signalling pathways implicated in the metastatic progression which includes the phosphorylation of MUC1 cytoplasmic domain by Epidermal Growth Factor Receptor (EGFR) that promotes cell motility and also the interaction with transcription factors, such as β-catenin, promoting the transcription of EMT genes[80].

Also, considering the MUC1, some studies show that, once in circulation, this glycoprotein can behave as an inhibitory factor in response to inflammation mediated by T-cells in cancer[107,108]. We tried to know whether these cell lines secreted MUC1, over the time, into the culture medium. The secreted form of MUC1 was not detected in cell culture supernatants, however, it may be necessary to repeat this assay because MUC1 molecular weight is considerable which makes its transfer less effective, on the other hand, it may be necessary to increase the protein concentration in the sample.

It has been shown that cells whose E-SL expression, such as the sialofucosylated $sLe^{x/a}$ ligands, is increased have higher proliferation rates and higher migration capacity[126], two fundamental features in cancer. In fact, our results show that fucosylation plays an important role in cell proliferation and migration. Cells treated with 2-FF showed lower proliferation rates as well as lower migration capacity. Furthermore, our data show that sialofucosylated ligands like $sLe^{x/a}$ play an important role in these two processes. In SW48 cell line, where expression of $sLe^{x/a}$ was not detected, the proliferation index and the migratory capacity, although affected by fucosylation, showed small variations. These results also help to understand, for example, the swift change in the CK expression pattern in HT29 cell line. Actually, these cells show significantly higher proliferation rates than the remaining cell lines, so, the next generations of these cells arise faster and exhibit the new expression pattern.

Considering the migration assays, our results are in accordance with the functionality of E-selectin and its ligands[62]. Indeed, when tumour cells initiate migration processes they need to express large amounts of $sLe^{x/a}$, so, later, they can recognize and bind to E-selectin in endothelial cells in order to initiate the invasion and metastasis processes. In this case, we have also shown that in cells where fucosylation was inhibited, and therefore the expression of $sLe^{x/a}$ decreased, the migratory capacity was lower.

Considering that the processes of proliferation and migration, in addition to being closely related to the tumour microenvironment, are closely related to the presence or absence of certain growth factors as well as signalling molecules like proinflammatory cytokines, important coordinators of inflammation[127,128]. Changes in the expression of some of these molecules are likely to occur. Our results showed that inhibition of fucosylation decreased expression of growth factors such as TGF-β, FGF2 and VEGF-A. In fact, growth factors are related to cell proliferation and migration, for example, TGF-β factor is often mutated in several types of cancer, allowing cell cycle progression and therefore, higher proliferation rates[129,130], whereas FGF2 and VEGF-A are closely related to tissue invasion and metastasis[131,132]. VEGF-A is a pro-angiogenic factor which in addition to inducing angiogenesis and endothelial growth, is also responsible for increasing endothelial permeability, facilitating tissue invasion[133,134]. Regarding proinflammatory cytokines, our results show that cancer cells are able to produce factors that increase proinflammatory contexts, since they express cytokines like IL-1β and IL-6. This observation consolidates the importance of inflammation in malignant transformation, hence it is considered one of the new Hallmarks of cancer[42].

On the other hand, the inhibition of fucosylation appears to induce two different contexts with respect to the expression of IL-1β once in LS174T and SW48 cell lines its expression decreases, whereas in HT29 cell line its expression increases, suggesting that, in these cells, fucosylation will be responsible

for increasing the inflammatory marker. In fact, there are studies that show a duality in the function of proinflammatory cytokines, namely in IL-1 family[111]. Moreover, there are studies that suggest regulatory networks in which IL-1β is able to neutralize the expression of certain proteins in acute inflammatory stages whose expression has been induced by IL-6[135,136]. In fact, in our results, HT29 cells subjected to treatment with fucosylation inhibitor exhibit elevated IL-6 expression which may explain, according to these studies, increased IL-1β cytokine expression.

Together, our results related to the expression of growth factors and proinflammatory cytokines, in addition to showing that our cell lines are highly proliferative and invasive, suggest that fucosylation plays a fundamental role in the functioning and modelling of certain signalling pathways. Actually, one of the Hallmarks of cancer is the ability of tumour cells to sustain their own proliferative signalling, producing their own growth factors and acquiring the capacity to avoid suppressor factors, which involves a constant modelling of signalling pathways activity[42].

Finally, we have attempted to study the EMT in these cell lines. For this analysis, we recovered the CF1T cell line since, for being a cancer cell line, will inevitably have an EMT stage during cancer progression for invasion and metastasis in distant organs. Our results showed that inhibition of fucosylation is responsible for reversing the expression patterns of CK and fibronectin, epithelial and mesenchymal markers, respectively.

In fact, cancer cells in the pre-migratory stages are characterized by increased expression of mesenchymal markers and decreased expression of epithelial markers, in the so-called EMT. This phenotype is characterized by a progressive loss of adhesion to the epithelium for entry into the bloodstream where cancer cells will migrate to invade new tissues[137,138]. Moreover, α1,3/4 FUTs have been reported to be involved in several tumour features, including the EMT[87,139]. Also increased proliferation rates as well as increased migratory capacity, are associated with an increase in fibronectin expression[140]. Our results are in agreement with these observations since the inhibition of fucosylation, which we have shown to decrease proliferation rates and migration, was also responsible for decrease the expression of fibronectin, returning epithelial properties to these cells.

5. Conclusion and Future perspectives

In this thesis, we identified aberrant glycosylation patterns in colon adenocarcinoma and TNBC tissues and characterize these tissues for the expression of E-SL and for potential proteins that can be decorated with such glycosylation patterns, suggesting MUC1 as a candidate. Then, using cell lines as a model, we found that fucosylation plays an important role in cancer progression and metastasis. Inhibition of fucosylation decreases the expression of sialofucosylated ligands, important players in the recognition of E-selectin, and increases CK expression as well as the underglycosylated form of MUC1, that is accumulated in the cytosol. The inhibition of fucosylation also decreases the expression of growth factors and proinflammatory cytokines and further reduces cell proliferation and migration. In addition, the inhibition of fucosylation decreases the expression of mesenchymal markers, suggesting a delayed EMT.

Taken together, our results, not only show the importance of E-SL in tumour development, but also suggest that inhibition of fucosylation may be effective in restoring an epithelial phenotype and therefore in decreasing cancer progression and metastasis.

In the future, some assays must be reviewed, namely the evaluation of $sLe^{x/a}$ expression in CF1T cell line and the investigation of the secreted form of MUC1 in the culture medium supernatant in order to identify possible functions at the tumour microenvironment. To deepen our results, we suggest a more in-depth evaluation of the CK expression patterns in tumour cells, initially a division between basal and luminal CK should be considered. Concerning MUC1, it would be important to determine the function of N-glycosylation in its folding and transport to the membrane, looking for a link between fucosylation, N-glycosylation and MUC1 accumulation in the cytosol. It would also be interesting to study the signalling pathways where cytoplasmatic cleaved tail of MUC1 is involved. Regarding EMT, other markers should be evaluated, namely Vimentin, β-catenin and E-cadherin. At the same time, a study of Wnt and Notch signalling pathways should be developed.

Finally, in order to complement our results, adhesion assays can be developed as well as a new model that allowed to study the reverse process of EMT, the mesenchymal-epithelial transition (MET), by which tumour cells need to pass when they establish in new tissues. Also studies of sialylation may be useful to better understand the effect of fucosylation in cancer progression and metastasis since $sLe^{x/a}$ is a sialofucosylated ligand. Regarding the tumour tissues, it would be interesting to study these markers in the metastasis and compare those results with primary tumours. New therapies may emerge from this comparative study.

In the future, it will also be necessary to analyse more cancer tissues, including other types of cancer, as well as increase the number of replicates to assess reproducibility and attribute more significance to our results. Further studies may contribute to the design of new therapies based on fucosylation.

6. References

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Appendices

Appendix 1 – Solutions

TBS 10X – 154 nM Tris-HCl; 1.37 M NaCl. All the reagents in dH_2O with pH 7.6

TBST 0.1% - TBST 1X; Tween 20

PBS 10X – 14.7 mM KH_2PO_4; 42.9 mM Na_2HPO_4; 1.37 M NaCl; 26.8 mM KCl. All the reagents in dH_2O with pH 7.4

Running Buffer 10X – 1.92 M Glycine; 0.25 M Tris-base; 35 mM SDS. All reagents in dH_2O.

Transfer Buffer 10X – 1.92 M Glycine; 0.25 M Tris base. All reagents in dH_2O.

6% Resolving Gel – 1.65 mL dH_2O; 2 mL 20% Acrylamide; 1.25 mL 1.5 M Tris (pH 8.8); 100 µL 10% SDS; 10 µL TEMED; 100 µL 10% APS (for 1 gel).

12% Resolving Gel – 3.3 mL dH_2O; 4 mL 20% Acrylamide; 2.5 mL 1,5 M Tris (pH 8.8); 100 µL 10% SDS; 10 µL TEMED; 100 µL 10% APS (for 1 gel).

4% Stacking Gel – 1.8 mL dH_2O; 840 µL 20% Acrylamide; 1.5 mL 0.5 M Tris (pH 6.8); 30 µL 10% SDS; 3 µL TEMED; 30 µL 10% APS (for 1 gel).

SHAPE	White (Generic)	Blue	Green	Yellow	Orange	Pink	Purple	Light Blue	Brown	Red
Filled Circle	Hexose	Glc	Man	Gal	Gul	Alt	All	Tal	Ido	
Filled Square	HexNAc	GlcNAc	ManNAc	GalNAc	GulNAc	AltNAc	AllNAc	TalNAc	IdoNAc	
Crossed Square	Hexosamine	GlcN	ManN	GalN	GulN	AltN	AllN	TalN	IdoN	
Divided Diamond	Hexuronate	GlcA	ManA	GalA	GulA	AltA	AllA	TalA	IdoA	
Filled Triangle	Deoxyhexose	Qui	Rha		6dGul	6dAlt		6dTal		Fuc
Divided Triangle	DeoxyhexNAc	QuiNAc	RhaNAc			6dAltNAc		6dTalNAc		FucNAc
Flat Rectangle	Di-deoxyhexose	Oli	Tyv		Abe	Par	Dig	Col		
Filled Star	Pentose		Ara	Lyx	Xyl	Rib				
Filled Diamond	Deoxynonulosonate		Kdn				Neu5Ac	Neu5Gc	Neu	Sia
Flat Diamond	Di-deoxynonulosonate		Pse	Leg		Aci		4eLeg		
Flat Hexagon	Unknown	Bac	LDmanHep	Kdo	Dha	DDmanHep	MurNAc	MurNGc	Mur	
Pentagon	Assigned	Api	Fru	Tag	Sor	Psi				

Supplementary Figure 1 - Symbol Nomenclature of Glycans[141].